

# Measurement and Treatment of Oxidative Stress in Chronic Obstructive Pulmonary Disease

David Graeme Anderson  
MBChB MRCP

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# Abstract

## Measurement and Treatment of Oxidative Stress in Chronic Obstructive Pulmonary Disease

Chronic Obstructive Pulmonary Disease (COPD) is a major cause of morbidity and mortality worldwide. Oxidative stress in the lung has been associated with the pathogenesis of this disease. Monitoring of the degree of oxidative stress and subsequent inflammation in the lung through non invasive collection of induced sputum and exhaled breath condensate (EBC) may improve our understanding of this disease process. Treatment to reduce oxidative stress in COPD may improve health status and lung function.

This thesis covers three studies. First of all the reproducibility of non invasive biomarkers was assessed and then a cross sectional study of these biomarkers was carried out. Finally a study of the impact of an inhaled anti oxidant on health status and non invasive biomarkers in subjects with COPD was carried out.

The reproducibility of differential cells counts and pro inflammatory cytokines IL-1 $\beta$ , IL-6, IL-8 and VEGF in induced sputum was assessed in 47 subjects. Total cell counts and macrophage differential counts were reproducible but not neutrophil and eosinophil differentials. IL-8 and VEGF but not IL-1 $\beta$  and IL-6 demonstrated reproducibility in induced sputum supernatant. Exhaled breath condensate was measured in 24 subjects. 8-Isoprostane but not hydrogen peroxide was reproducible.



Exhaled breath condensate was collected in 78 with COPD and 61 controls.

Groups were subdivided into current and ex-smokers. Levels of 8-Isoprostane and hydrogen peroxide were measured. No significant differences were seen between the mean levels of these two biomarkers measured in COPD and control groups. Levels of oxidative stress biomarkers were compared to health status, symptom scores and lung spirometry in the COPD population. No significant associations were noted in the current smokers. COPD ex-smokers from the lowest quartile (Q1) of 8-Isoprostane measured had lower health status and exacerbation frequency compared to the highest quartile (Q4). Lung function was worse in the highest 8-Isoprostane quartile. Hydrogen peroxide levels in EBC did not relate to health status or symptom scores.

Fifty-eight subjects with moderate to severe COPD participated in a 12 week double blind placebo controlled trial of an inhaled lyseine salt of N-Acetylcysteine. Fewer of the subjects in the low dose treatment arm of the study had clinically significant exacerbations compared with placebo. The low dose treatment arm also demonstrated improvements in terms of diary card reporting of breathlessness when compared with placebo.

In summary, some non invasive biomarkers of oxidative stress in COPD are reproducible. However the overall utility of EBC 8-Isoprostane and hydrogen peroxide measurement in COPD appears limited. 8-Isoprostane levels in ex-smokers with COPD may reflect disease activity. Treatment with low dose inhaled antioxidant demonstrated some improvement in health status.

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# Declaration

I declare that this thesis has been composed by myself, that the work is my own and that the work has not been submitted for any other professional qualification.

David Graeme Anderson

1<sup>st</sup> August 2007

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# Publications and Presentations arising from this Thesis

## PUBLISHED PRESENTATIONS

- Sep 2003      Reproducibility of Biomarkers of Oxidative Stress and Airways Inflammation in Induced Sputum and Exhaled Breath Condensate  
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American Thoracic Society 2004
- Sep 2004      Biomarkers in breath condensate and induced sputum in COPD- relationship to FEV<sub>1</sub>, BMI and health status  
D. G. Anderson, M. McLaughlin, R. Frangulyan, E. Drost, W. MacNee  
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- Sep 2005      Health indicators and inflammation: a comparison of stable smoking and ex-smoking COPD patients  
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## PAPERS IN SUBMISSION

Biomarkers of Oxidative Stress in Breath Condensate of COPD and Controls: Relationship with Spirometry and Health Status

D.G. Anderson, M. McLaughlin, J. Miller, C. Poland, E. Drost and W. MacNee

Role of inhaled antioxidants in COPD

D Anderson, M McLaughlin, J Barr, A Deans, C Poland, C McGuinness, E Drost, K Donaldson and W MacNee

# List of Abbreviations

$\alpha$ 1-AT	$\alpha$ 1Antitrypsin
4-HNE	4-hydroxy nonenal
AM	Alveolar macrophages
BAL	Broncho-Alveolar Lavage
BD	bronchodilator
BMI	Body mass index
COPD	Chronic Obstructive Pulmonary Disease
CoQ-10	coenzyme Q-10
CRP	C Reactive Protein
DTT	dithiothreitol
EBC	Exhaled Breath Condensate
ELF	Epithelial lining fluid
ET	Early Termination
FEV <sub>1</sub>	Forced Expiratory Volume in 1 sec
FEV <sub>1</sub>	Forced Expiratory Volume in 1 second
FVC	Forced Vital Capacity
GSH	reduced glutathione
GSSG	oxidised glutathione
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
HO	Haem Oxygenase
HOCl	Hypochlorous acid
ICAM-1	Intracellular adhesion molecule 1
ICS	Inhaled corticosteroids

ICS	Inhaled corticosteroid
IκB	Inhibitory κB
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
IS	Induced Sputum
L	Litre
LABD	Long acting bronchodilator
LTB-4	Leukotriene B4
MAP-Kinase	Mitogen-activated protein kinases
MCP-1	monocyte chemotactic protein
MDA	Malondialdehyde
MID	Minimal Important Difference
MMP-9	Matrix metalloproteinase 9
MPO	Myeloperoxidase,
NAC	N-acetylcysteine
NADPH	nicotinamine adenine dinucleotide phosphate
NAL	Nacystelyn
NE	neutrophil elastase
NF κB	Nuclear Factor κB
PBS	phosphate buffered saline
PGE2	Prostaglandin E2
RNS	reactive nitrogen species
ROS	Reactive oxygen species
SD	Standard Deviation
SEM	Standard Error of Mean
SGRQ	St Georges Respiratory Questionnaire.

SLP-1	Secretory Leukoprotease-1
TBARS	Thiobarburic acid reactive substances
TGF $\beta$ 1	Transforming Growth Factor $\beta$ 1
TNF $\alpha$	Tumour Necrosis Factor $\alpha$
VEGF	Vascular Endothelial Growth Factor
XO	Xanthine oxidase

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# Chapter 1

## Introduction

# Chapter 1 Introduction

## 1 Overview

Chronic Obstructive Pulmonary Disease (COPD) is a common condition which presents later in life with exertional breathlessness and cough. COPD is strongly associated with cigarette smoking, although other factors can contribute to its development. The disease has a major impact on the lives of patients and their carers, health care services and society as a whole. The burden of disease and mortality of this condition are predicted to rise.

The pathogenesis of COPD is associated with a variety of inhaled toxins, including cigarette smoke. One consequence of this is an increased burden of free radicals in the lung creating an oxidant / antioxidant imbalance resulting in oxidative stress. Increased oxidative stress is thought to enhance inflammation responses both in the lung and systemically. Assessment of this process should assist in our understanding of the disease. Interventions to reduce the oxidative stress may decrease levels of inflammation in the lungs with the potential to improve morbidity and mortality in COPD.

The introduction of this thesis will include a summary of the epidemiology of COPD along with the clinical features and management of this condition. The underlying mechanisms associated with the pathogenesis of COPD will be discussed along with methods of assessing these mechanisms. Finally previous trials of anti-oxidant therapy in COPD will be reviewed.

## **1.1 ERS/ATS Consensus Statement - Definition of COPD**

“Chronic obstructive pulmonary disease (COPD) is a preventable and treatable disease state characterised by airflow limitation that is not fully reversible.

The airflow limitation is usually progressive and is associated with an abnormal inflammatory response of the lungs to noxious particles or gases, primarily caused by cigarette smoking.

Although COPD affects the lungs, it also produces significant systemic consequences.”(1)



## **1.2 Epidemiology and Risk Factors in COPD**

Cigarette smoking has the strongest association with the development of COPD although a variety of other factors have also been implicated. It is uncommon for patients with COPD to present under the age of 40 (2). As COPD presents later in life, identification of agents, both endogenous and exogenous, which are implicated in the pathogenesis of COPD is problematic. Subjects will have been exposed to a multitude of environmental risk factors throughout their lives which may have had a role in the development of COPD at the time of presentation. The innate susceptibility of each individual to this environmental exposure is also important in the development this disease.

For the purpose of this review, risk factors have been divided into those which are directly harmful to the lungs and those which affect the way the host responds to these initial insults.

### **1.2.1 Inhaled Toxins**

#### **1.2.1a Cigarette Smoke**

The association between COPD and smoking dates back to the 1950s when Fletcher et al demonstrated a clinical relationship between smoking and cough and sputum production(3, 4).Pathological studies have also demonstrated a relationship between smoking, mucus hypersecretion and subsequent development of chronic bronchitis and emphysema(5). Mucus hypersecretion is

associated with a decline in Forced Expiratory Volume in 1 second ( $FEV_1$ ), a measurement of airflow limitation, in patients with COPD independent of age, height, weight change and smoking(6).

Relationships have also been shown between smoking and airflow limitation and also on the impact of smoking on the rate of decline of  $FEV_1$ . In a recent open survey in Poland of patients older than 40 years of age with no previous history of asthma, bronchiectasis or tuberculosis, a higher percentage of subjects who are current or former smokers have evidence of airflow limitation on spirometry (23%) compared with lifelong non smokers (12%). A trend has also been demonstrated between worsening airflow limitation and increasing smoking history(7). On average ex-smokers have higher  $FEV_1$  values than current smokers(8).

A strong relationship has been reported between cigarette smoking and the rate of decline of  $FEV_1$ (9), with non smokers losing on average 25ml of  $FEV_1$  per year compared with 50ml per year in smokers and 100ml per year in heavy smokers(4). Cross-sectional data indicates that quitting smoking is associated with reduced rate of decline in  $FEV_1$ (9, 10). The association between the rate of decline in  $FEV_1$  and smoking is independent of the maximum obtained lung function in adolescence(11). There is also some evidence that exposure to passive smoking may have an impact on the rate of decline of  $FEV_1$ , in the region of 3.8ml/year(12). The Lung Health Study demonstrated that there is a significant variation in the rate of decline of  $FEV_1$  in smokers with COPD(13), the "rapid declining" falling by 150ml per year compared to 15ml per year in the "non declining" cohort.

Despite this strong evidence of the effect of smoking on development of chronic bronchitis and airflow limitation, the relationship is not absolute. COPD is present among lifelong non-smokers(14), more than 15% of subjects worldwide who die from COPD are non smokers. Less than 25% of subjects who have a significant smoking history go on to develop COPD.

### **1.2.1b Environmental Toxins**

Other inhaled environmental agents have been identified in the development of COPD. A link exists between high levels of indoor air pollution and the incidence of COPD(15). Furthermore increased outdoor particulate pollution levels result in increased mortality in subjects with COPD, both in COPD deaths and deaths from other causes(16). Several occupational agents have also been implicated in COPD pathogenesis. These include welding fumes, grain, isocyanates, cadmium and coal dust(17)..

### **1.2.2 Host Response**

The fact that the relationship between smoking and COPD is not absolute highlights the fact that it is not simply the inhaled insult that results in the development of COPD; the host response to the inhaled insult also plays a major role. Several factors have been implicated in the host response including genetics, health and illness in early life, environmental factors, nutrition and bacterial colonisation.

### 1.2.2a Genetics of COPD

The strongest evidence of a genetic relationship in COPD is the deficiency of the anti-protease  $\alpha$ 1-antitrypsin. Deficient release of this his molecule results in early development of COPD. ZZ homozygotes produce very low levels of  $\alpha$ 1-antitrypsin. These patients have an Odds Ratio greater than 30 of developing COPD(18). Milder forms of this deficiency also have an increased incidence of COPD, MZ heterozygotes for example have an odds ratio between 1.5 and 5 of developing COPD(19). Overall however the incidence of  $\alpha$ 1AT deficiency in the COPD population is low, and is implicated in less than 1% of patients with COPD(20).

Genetic factors other than  $\alpha$ 1-antitrypsin deficiency exist. The relationship between these factors and COPD development is not as strong as  $\alpha$ 1-antitrypsin. Furthermore there is a significant variation in the strength of these relationships between populations.

Genes which have been implicated include glutathione-s-transferase polymorphism(21),  $\alpha$ 1-antichymotrypsin(22, 23). Genetic predisposition may also be associated with the  $\text{TNF}\alpha$  gene which exists in two forms, the  $\text{TNF}\alpha$ 2 iso-form is associated with increased levels of chronic bronchitis(24).

### 1.2.2b Development

Health in early life appears to be a major factor in the development of COPD. An association exists between maximum obtained lung function and subsequent development of COPD in later life. Factors which influence maximum obtained function include birth weight, time of gestation and fetal and peri-natal smoke exposure(25-27). Sub-optimal lung function in adolescence is associated with subsequent development of COPD(28). Airways hyper-responsiveness in early adulthood is associated with subsequent development of COPD(29). Smokers with airways hyper-responsiveness have been shown to develop COPD more rapidly than smokers with no airways hyper-responsiveness(30).

#### **1.2.2c Nutrition**

Nutrition also appears to have a role in development of COPD. Low fresh fruit intake is associated with lower FEV<sub>1</sub>(31). Poor nutrition as defined by Body Mass Index (BMI) is also associated with COPD. Weight loss or a low BMI is associated with COPD mortality, this relationship being strongest in severe COPD(32, 33)and is independent of weight or sex(34). Development of airflow limitation is associated with reduced dietary vitamin C intake(35, 36) whilst high intake of fruit and whole grains appears to protect against declining lung function(31).

#### **1.2.2d Gender**

Gender may also be a factor in COPD pathogenesis, independent of smoking consumption, with females at higher risk of admission with COPD compared with matched males(37).

### **1.2.2e Socioeconomic Status**

Socioeconomic status appears also to play a role, with subjects from the lowest end of the socioeconomic spectrum having between 200 and 400ml lower mean FEV<sub>1</sub> values compared with the highest end, independent of other risk factors including smoking(38). Admission rates have also been noted to be higher in subjects with lower socioeconomic status(1).

### **1.2.2f Bacterial Colonisation**

Bacterial colonisation in COPD is associated with declining FEV<sub>1</sub>. Increased bacterial load is associated with increased rate of decline in FEV<sub>1</sub>(39). Subjects with severe COPD have increased frequency of exacerbations of COPD compared with milder forms of the disease(40).

### 1.2.3 Prevalence and Incidence of COPD

There is a large variation in published data on the prevalence of COPD. One reason for this is that a proportion of the population will have evidence of airflow limitation on spirometry without any symptoms consistent with COPD. Prevalence studies which screen for airflow limitation independently of symptoms will therefore demonstrate a higher prevalence compared with studies which require both spirometric and clinical features of COPD.

The prevalence of chronic bronchitis varies throughout Europe, averaging 4% in Europe, ranging from 3.2% to 5.4%(41). In the United States of America, the National Health Interview Study in 1996 demonstrated 6% of the population over the age of 25 reporting that they suffered from chronic bronchitis or emphysema(42). In 1997 5.9% of the US population reported that they had physician diagnosed chronic bronchitis or emphysema(42).

Airflow limitation alone is more prevalent than symptoms of chronic bronchitis. In the United Kingdom 10% of males and 11% of females aged between 18 and 65 had FEV<sub>1</sub> values at least 2 standard deviations below predicted values for age and height(43). 33.5% of patients with significant airflow limitation do not report any respiratory symptoms(7). This is more apparent at higher levels of FEV<sub>1</sub>. Lower levels of FEV<sub>1</sub> are associated with increase rates of admission and also increasing respiratory symptoms(44).

In the US, the National Health and Nutrition Examination Survey III (NHANES III) demonstrated that 14.3% of the adult population had spirometric abnormalities consistent with COPD. However in association with physician diagnosed emphysema or chronic bronchitis this dropped to 2.9% of the population. Only 1.4% of the population had an FEV<sub>1</sub> less than 50% predicted. 71.7% of subjects with mild airflow limitation had no formal diagnosis of COPD(45).

Worldwide prevalence of COPD is lower than levels reported in Europe and the United States, reflecting higher cigarette consumption in these countries. The GOLD report estimated the global prevalence of COPD in 1990 to be 9.34 per 1000 men and 7.33 per 1000 females of all ages(46).



#### 1.2.4 Morbidity and Mortality of COPD

The morbidity associated with COPD can be quantified in several ways, including the impact on individuals, the health service and society as a whole.

COPD has a major impact on the individual. In the UK 73% of patients with physician diagnosed COPD reported that they were unable to undertake important activities due to their condition and the forty-five percent with a clinician diagnosis of COPD, emphysema or Chronic Bronchitis were either unable to work or had stopped work early because of COPD.(47).

Management of COPD is a major consumer of health services worldwide, in terms of out-patient review, emergency consultations and hospital admissions. Two recent surveys in the US have established that COPD is responsible for 82 ambulatory visits per 1 000 population per year(48), and 8.3 visits to emergency room per 1 000 of the population per year(49). COPD also has a large impact in terms of hospital admissions, a recent survey of US hospital admissions in 1998 demonstrated 38.3 admissions per 10 000 of the population per year. These admissions accounted for 1.9% of the total hospital admissions. A further 7.0% of total hospital admissions identified COPD as contributing to hospitalisation(50, 51). A recent telephone survey of the impact of COPD demonstrated that 12.8% of diagnosed patients had been hospitalised in the last year due to exacerbations of

COPD(41). The number of hospital admissions due to exacerbations of COPD is increasing(52).

Disability adjusted life years can be used to express the overall burden of a disease on society. The World Health Organisation (WHO) estimated that 2.1% of all disability life years were as a result of COPD in 1990, the 12<sup>th</sup> most prevalent cause of years lost due to disability. This value is predicted to rise to 4.1% in 2020, ranking as the 5<sup>th</sup> largest cause of disability adjusted life years(53).

The mortality related to COPD is also predicted to rise during the same timeframe. The WHO ranked COPD as the 6<sup>th</sup> most frequent cause of mortality in 1990 and estimates that this will increase to 3<sup>rd</sup> by the year 2020(54). This increase appears to be predominately in females. Current death rates in the US are 53.1 per 100 000 males per year which is a stable figure and 32.1 per 100 000 females, a figure which is rising(55). Between the years 1974-1993 a 126% increase in female mortality due to COPD was recognised in the US(56). A similar increase in mortality in females has been reported in Australia, where a 2.6 fold increase in female mortality was noted between 1964 and 1990(57). Overall 8.2% of all patients dying in the US had obstructive lung disease listed as a cause(56).

Mortality figures in Europe are similar to those in the US, although a large variety is seen between countries, ranging from greater than 30 per 100 000 of the population in Hungary and Denmark(58, 59) to less than 10 per 100 000 population in Spain and France(60).

## 1.3 Clinical Features

### 1.3.1 Clinical History

Chronic Obstructive Pulmonary Disease is characterised by chronic respiratory symptoms of breathlessness, cough, sputum production and wheeze.

Breathlessness is often progressive with a gradual decline in symptoms over many years. In a similar fashion cough may initially be mild and only present first thing in the morning, i.e. a “smoker’s cough”, then gradually become productive, persist throughout the day and increase in severity.

Patients normally present later in life, usually over the age of 40, unless there is a genetic predisposition to the disease such as  $\alpha 1$  antitrypsin deficiency. In the majority of cases there is a significant history of exposure to cigarette smoke.

Patients with COPD are prone to acute exacerbations of their disease. These exacerbations can be precipitated by viral or bacterial lower respiratory tract infections. Increased levels of air pollution are also associated with some exacerbations. Exacerbations which result in hospital admission are associated with a high mortality.

Subjects with COPD may go on to develop respiratory failure. Clinical symptoms of right sided heart failure such as ankle oedema may be noted. Chronic type II respiratory failure with carbon dioxide retention may result in symptoms of intermittent drowsiness and confusion.

There is a wide clinical spectrum in COPD. This ranges from mild airflow limitation without symptoms late in life, to a more aggressive disease with recurrent hospital admissions and premature death.

Historically patients with smoking-related lung disease were previously diagnosed as having chronic bronchiolitis, chronic bronchitis or emphysema, thought to be separate disease processes. Chronic bronchitis is defined clinically as a cough productive of sputum most days for 3 consecutive months for two consecutive years. Emphysema is a pathological diagnosis dependant on evidence of enlargement and destruction of alveolar walls distal to the terminal bronchioles. Chronic bronchiolitis is defined pathologically as chronic inflammation of the peripheral airways. This occurs early in the disease and is often an asymptomatic finding (61). Matsuba et al demonstrated that the internal diameter of respiratory and membranous bronchioles in subjects with a FEV<sub>1</sub> greater than 75% correlates with the FEV<sub>1</sub>(62). A similar relationship is present with more significant airflow limitation(63).

There is a large clinical overlap between these conditions. Furthermore they are frequently characterised by breathlessness and progressive airflow limitation. This led to the introduction of the term Chronic Obstructive Pulmonary Disease encompassing chronic bronchiolitis, chronic bronchitis and emphysema.

Differentiation from other obstructive airways diseases such as asthma can sometimes be problematic. Salient points in the history include character, duration and onset of symptoms, history of atopy, smoking history and family history. Sudden deterioration and variability in symptoms with obvious triggers are more characteristic of asthma. Predominant nocturnal symptoms are also more likely in asthma. A history of atopy or childhood asthma would make the diagnosis of asthma more likely, as would a family history of asthma. Presentation under the age of 40 in a non smoker is unusual in COPD.

### **1.3.2 Clinical Examination and Investigation**

Clinical examination is of limited usefulness in the diagnosis of COPD, with many clinical findings being non specific. Salient points on examination include tachycardia, tachypnoea and hyperinflation of the lungs with reduced chest expansion and air entry, with or without wheeze.

Respiratory failure as a consequence of COPD may result in abnormalities on clinical examination. Carbon dioxide retention can cause a bounding pulse, papilloedema and a flapping tremor. Evidence of right sided heart failure as a

consequence of respiratory failure may be noted with right ventricular hypertrophy and peripheral oedema. ECG changes demonstrating right sided hypertension and right ventricular hypertrophy may also be apparent.

Oxygen saturations should be assessed by pulse oximetry and arterial blood gas measurement made if there is evidence of hypoxia. Nutritional assessment should be made by measuring the Body Mass Index.

Chest Radiographs may demonstrate evidence of emphysema. If the condition is severe, emphysema can also give rise to bullae, hyperinflation of the lung with flattening of the diaphragms.

As stated above, COPD is characterized by chronic airflow limitation. Spirometry measures the volume of air which can be expired by in one second (forced expiratory volume in one second, FEV<sub>1</sub>) and the total lung volume which can be exhaled, the forced vital capacity, FVC. The ratio between these two values defines the presence of airflow limitation. Subjects with airflow limitation have reduced airway calibre and consequently the rate at which a subject's lung volume can be exhaled is reduced. This causes less lung volume being exhaled in the first second

when compared with healthy individuals. The spirometric definition of COPD therefore includes a reduced FEV<sub>1</sub> and an FEV<sub>1</sub> / FVC ratio less than 0.7.

The severity of COPD can be defined using lung function testing. Mild, moderate, severe and very severe airflow limitation is then defined according to the level of FEV<sub>1</sub> compared with the percent predicted for that individual. Percentage predicted values take into account the height, sex, age and race of the subject. A variety of formulae are used to allow calculation of percentage predicted values, the European Coal and Steel Community formulae are frequently used in the UK(64)

Measurement of airflow obstruction before and after administration of an inhaled or nebulised bronchodilator is commonly used to assess the degree of reversibility of airflow obstruction. Subjects with asthma are more likely to have evidence of bronchial lability compared with patients with COPD. The use of reversibility as a diagnostic tool in COPD however is becoming less common. The recently published NICE guidelines have not included reversibility testing in routine COPD investigation(65)

### **1.3.3 Management of COPD**

Current therapy for COPD includes inhaled bronchodilators, both short and long acting. Short acting bronchodilators may also be given in nebulised form. These

therapies can improve health status and symptomatic control but have no impact on mortality(66-69). Inhaled corticosteroids (ICS) also have a role in COPD and can improve health status and reduce exacerbation rates(70, 71). Inhaled corticosteroid therapy in combination with long acting bronchodilators have been shown to be of greater benefit than ICS alone (72, 73). Treatment with ICS is currently recommended for subjects with an FEV<sub>1</sub> less than 50% who have more than one exacerbation per year(1). Treatment with oral mucolytics may reduce the number of exacerbations and is discussed in detail later in this chapter. Vaccination against influenza virus and streptococcus pneumoniae is also recommended(74, 75).

Subjects with COPD should be assessed for respiratory failure. Long term oxygen therapy is of benefit in subjects with significant respiratory failure and can improve mortality and morbidity(76, 77). There is also evidence that pulmonary rehabilitation improves mortality and morbidity in selected patients with COPD(78).

Other management considerations in COPD include assessment for genetic predisposition in those subjects who present earlier in life, nutritional supplementation, CT scanning to assess suitability for surgery such as bullectomy, lung volume reduction surgery and also suitability for referral for single lung transplant.

To summarise, COPD is diagnosed clinically from symptoms and confirmed by lung function testing. Clinical examination and chest radiography may aid in the diagnosis. The mainstay of management is symptomatic treatment with inhaled therapy as no pharmacological treatments have been shown to improve mortality.



## 1.4 Pathogenesis of COPD

Epidemiological evidence indicates that cigarette smoking and inhalation of other toxins contributes to the development of COPD. The response of the body to these inhaled toxins plays a crucial role in the pathogenesis of this disease. Various mechanisms have been implicated in this process. One hypothesis is that inhalation of toxins rich in free radicals results in oxidative stress in the lung. The lung responds to this oxidative stress by producing pro-inflammatory cytokines with a resultant influx of inflammatory leukocytes. The subsequent inflammation damages lung tissues, generates further oxidative stress and causes an imbalance between protease and antiprotease enzymes in the lung with further lung tissue damage.

### 1.4.1 Sources of Oxidative Stress

Oxidative stress results from an imbalance between oxidants and antioxidants in favour of oxidants. This can occur from either an increase in oxidant burden or a decrease in antioxidant or both. An increase in oxidant burden in the lungs can result from inhalation of toxins such as cigarette smoke or air pollution which generate oxidants, or because of inflammation in the lung. In the majority of patients the development of COPD is a consequence of chronic cigarette smoking. Cigarette smoke contains a variety of agents which are toxic to the lung. It is estimated that cigarette smoke contains  $10^{17}$  free radicals per inhalation(79) Also present in tobacco smoke are high concentrations of reactive oxygen species (ROS), both non-radicals such as hydrogen peroxide and oxygen radicals such as superoxide ion(80) and reactive nitrogen species (RNS) such as nitric oxide (NO.) which is present in smoke in concentrations of 500-1000 parts per million(81). Tobacco smoke contains aromatic hydrocarbons, such as benzene, which are also toxic to the lungs(80).

Chronic inhalation of cigarette smoke results in a significant oxidant burden for the lungs. It has been hypothesised that the ability of the lungs to cope with this oxidant burden is a important role in the development of COPD(82).

Smoke inhalation is not the only cause of oxidative stress in the lungs. Alternative sources of oxidative stress can be either exogenous such as from air pollution (83) or endogenous from inflammatory cells in the airways. Neutrophils, macrophages,

eosinophils and structural epithelial cells have all been identified as further potential sources of oxidants (84).

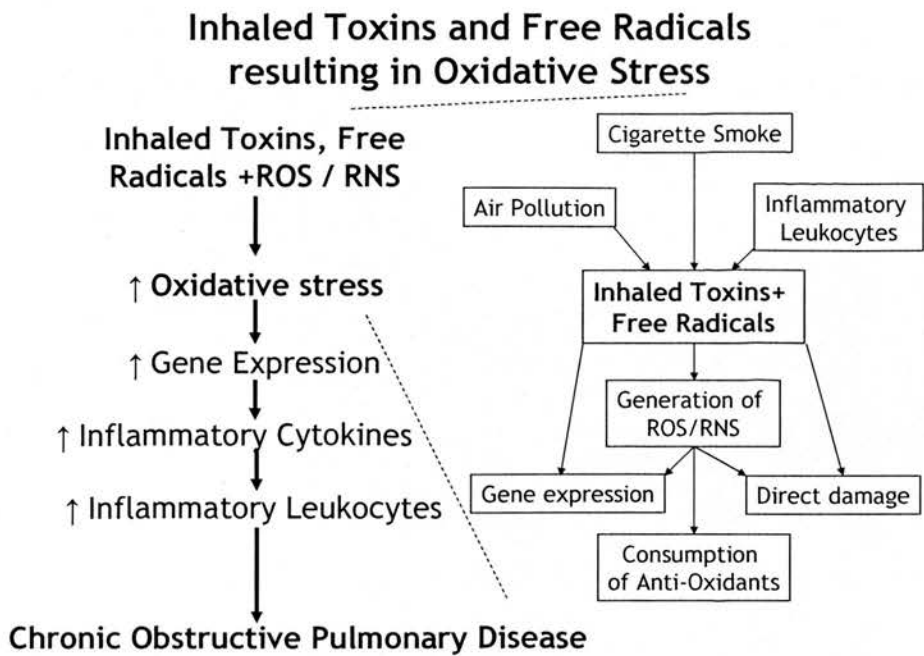


Figure 1.4.1 Oxidant Burden in the Lung



### 1.4.2 Generation of Reactive Oxygen Species

The presence of free radicals in the lungs results in generation of reactive oxygen species and reactive nitrogen species, which can lead to lung damage.

Free radicals in the lungs interact with oxygen to produce superoxide ion, figure

1.4.2. The nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system is a major source of superoxide production from inflammatory cells in the lung<sup>1</sup>.

Other sources of superoxide production are mitochondrial metabolism, arachidonic acid metabolism and molybdenum hydrolase reactions within cells (85, 86).

Superoxide is metabolised enzymatically by superoxide dismutase to produce hydrogen peroxide, figure 1.4.2. Superoxide ion and hydrogen peroxide can combine via the Fenton reaction to produce hydroxyl radicals (60, 84). Iron is the major catalyst of this reaction. Furthermore it has been shown that there is an excess of iron in the alveolar macrophages of smokers(87). In the lungs, granulocyte peroxidases, such as myeloperoxidase, metabolise hydrogen peroxide and chloride ions producing hypochlorous acid (HOCl).(88) Alternatively hydrogen peroxide can be dismuted by the enzyme catalase to water(89).

Superoxide ion and hydroxyl radicals are unstable molecules with a very short half life, making direct quantification of these molecules activity in vivo problematic.

Hydrogen peroxide is a stable compound which can be measured in Broncho-Alveolar Lavage (BAL) and exhaled breath condensate. Studies have shown elevated levels of hydrogen peroxide in expired breath of patients with COPD compared with healthy smokers and non smokers(90), with hydrogen peroxide rising further during exacerbations(91).

In summary free radicals in the lung interact with oxygen resulting in the production of the highly toxic molecules such as superoxide ion and hydroxyl radicals. These can be metabolised to hydrogen peroxide which itself is toxic to the lung. Hydrogen peroxide can be dismuted to water or can react with chloride resulting in the production of hypochlorous acid.

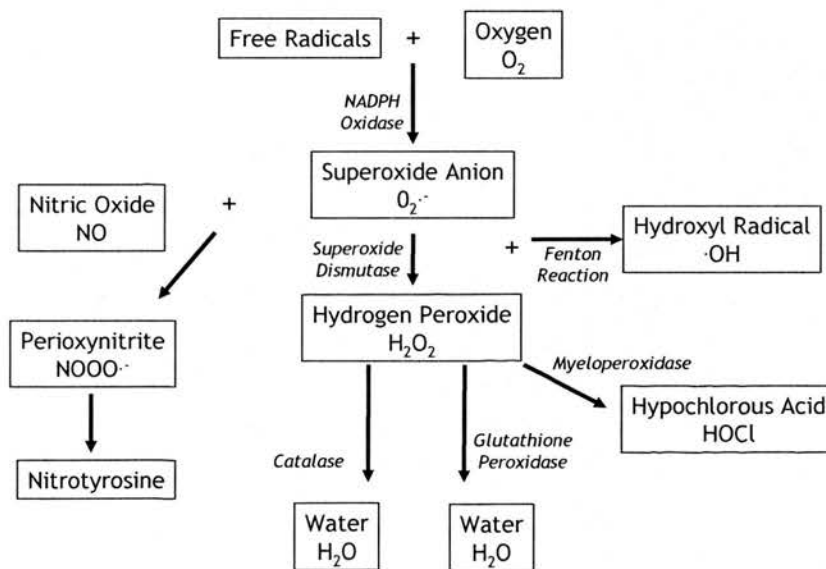


Figure 1.4.2 Free Radical, Reactive Oxygen Species and Reactive Nitrogen Species

### 1.4.3 Generation of Reactive Nitrogen Species

Production of reactive oxygen species (ROS) in the lung results in direct damage and inflammation. Along with these processes, which are discussed in section 1.4.2, ROS can interact with nitric oxide producing toxic molecules known as reactive nitrogen species (RNS).

Production of nitric oxide (NO) is a major factor in the generation of reactive nitrogen species. There are three iso enzymes involved in the production of nitric oxide, these being constitutive, inducible and neuronal nitric oxide synthase(92, 93). The cytokines TNF- $\alpha$  and IL-1 $\beta$  stimulate inducible nitric oxide synthase resulting in increased production of nitric oxide(94). At low concentrations nitric oxide is thought to be a signalling molecule(95). Increased levels of exhaled nitric oxide in smokers are associated with tobacco smoke lung damage(96).

Measurement of nitric oxide in COPD is controversial with contradicting studies indicating that NO is either elevated or reduced in this disease (97-99). Rutgers et al measured the inducible Nitric Oxide iso-enzyme (iNOS) in sputum macrophages with no difference seen between COPD and control(100). However, in subjects with COPD the number of pneumocytes with iNOS present in the alveolar wall of the lung parenchyma is elevated when compared with control subjects(101). Acute inhalation of cigarette smoke converts tyrosine to 3-nitrotyrosine and dityrosine,

this reaction can be inhibited by antioxidants such as glutathione, ascorbic acid and uric acid(102).

Nitric oxide at higher concentrations can both have a direct toxic effect(103) and interact with superoxide ion resulting in production of peroxynitrite(89) which reacts with tyrosine to produce 3-nitrotyrosine(104).

Peroxynitrite is not a stable compound and cannot be directly quantified. 3-nitrotyrosine is a product of peroxynitrite and can be measured by immunohistochemistry. Increased levels of 3-nitrotyrosine are demonstrated in sputum leukocytes of patients with COPD compared with control subjects(105, 106).

To summarise, there is evidence of increased reactive nitrogen species in COPD compared with controls, produced by reactions between NO and reactive oxygen species. Furthermore production of NO may be increased by oxidative stress in the lung.

#### **1.4.4 Other sources of oxidative stress**

In addition to the production of reactive oxygen and nitrogen species mentioned above, cigarette smoke contains many aromatic compounds which can cause damage to the lung.



Metabolism of these xenobiotics by cytochrome p450 results in the production of epoxides which are highly reactive and toxic to lung tissue. These epoxides are metabolised by conjugating enzymes such as glutathione-s-transferases epoxide hydrolases, which neutralise these toxic metabolites(107). Polymorphisms of microsomal epoxide hydrolase gene which are associated with reduced metabolism of epoxides are more commonly expressed in subjects with COPD compared with control subjects(108).

### 1.4.5 Effects of Oxidative Stress

Inhalation of toxic particles and gases in cigarette smoke and the subsequent oxidative stress can cause direct damage to lipids, proteins and DNA in the lungs(109, 110). Various mechanisms are involved in this process, figure 1.4.3. These include increased proteolytic degradation, impaired protective mechanisms and impaired cell membrane function.

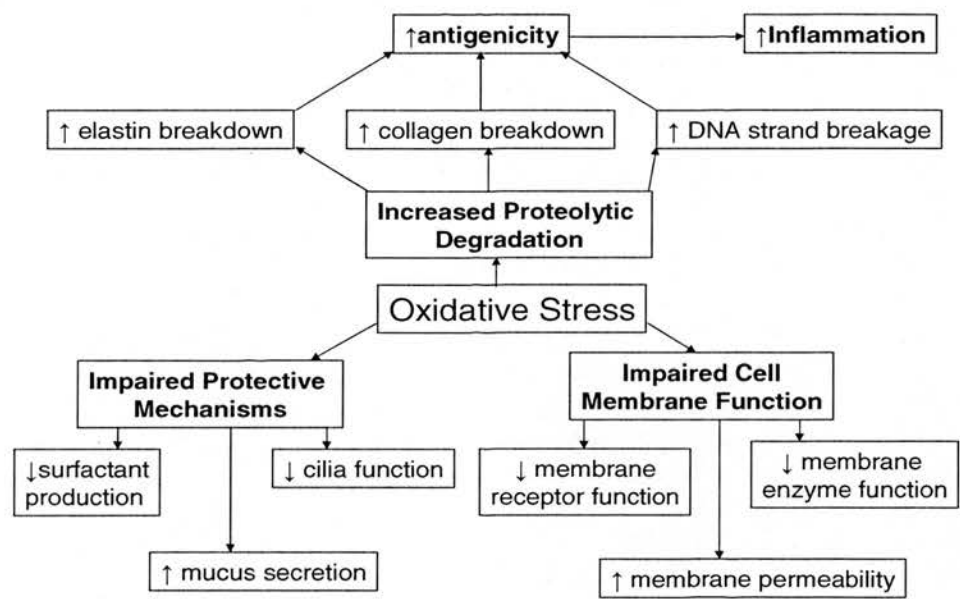


Figure 1.4.3 Effects of Oxidative Stress

The lung parenchyma and airways can be permanently damaged directly by oxidative stress(111) with fragmentation of elastin and collagen(112) along with reduced production of these structures.

Human respiratory tract epithelial cells treated with gas phase cigarette smoke show increased DNA strand breakage(113). Oxygen radicals in the lung cause modification to amino acid side chains resulting in cleavage of peptide bonds, resulting subsequently in proteins that are more susceptible to proteolytic degradation(114, 115). This direct damage can cause increased inflammation in the lung(116).

Reactive oxygen species, such as hydrogen peroxide and hydrochloric acid, impair host protective mechanisms with resultant damage to the lung. Oxidative stress in the lung results in impairment of cilia function(117), increased levels of high molecular weight mucus production(118) and increased epithelial permeability(119). Cigarette smoke inhalation also results in cell injury and reduced surfactant activity in fibroblasts(120).

Cell membrane function is also impaired by oxidative stress. Free radicals remove hydrogen atoms from side chains of lipids producing lipid radicals which react with oxygen to give peroxy radical. This action results in polyunsaturated fatty acids changing to lipid hydroperoxides(121). Lipid hydroperoxides have numerous toxic effects on the lung, including impaired membrane function, inactivation of membrane bound receptors and enzymes, and increasing epithelial permeability(122). Lipid hydroperoxides also interact with antioxidants or metal ions producing hydrocarbon gases and unsaturated aldehydes(123).

### 1.4.6 Oxidative Stress and Gene Expression

Section 1.4.5 discusses the process with which the lung is directly damaged as a result of oxidative stress. In addition to this direct damage, molecular pathways are stimulated by oxidative stress(124) resulting in increased gene expression, production of pro-inflammatory cytokines, influx of inflammatory leukocytes and subsequent enhanced inflammation in the lung.

#### Increased Gene Expression and Production of Pro-Inflammatory Cytokines in COPD

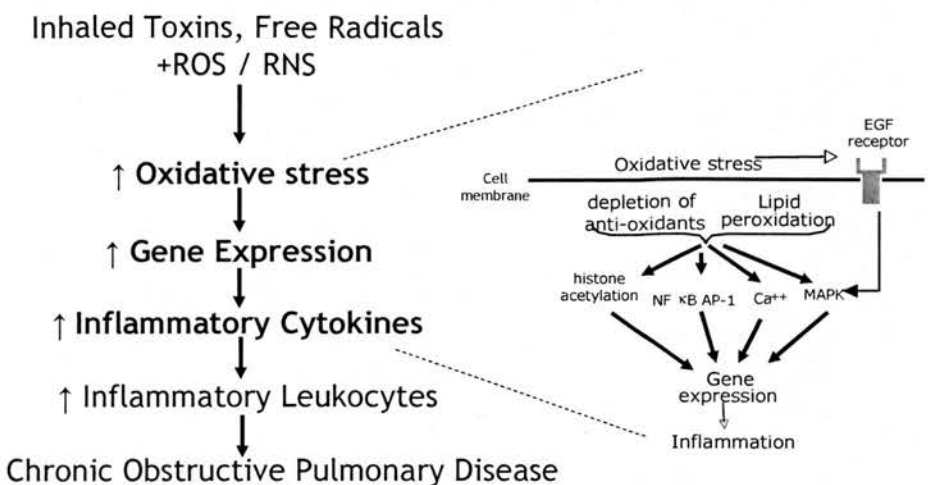


Figure 1.4.4 Oxidative stress and gene expression

The transcription molecule Nuclear Factor  $\kappa$ B appears to have an important role in oxidative stress induced gene expression of inflammatory mediators such as

cytokines, chemokines, adhesion molecules and inflammatory mediators(125, 126). ROS/RNS have been shown to activate NF  $\kappa$ B(127) via an enzyme dependant pathway(128, 129).

NF $\kappa$ B is normally bound to its inhibitory protein Inhibitory  $\kappa$ B (I $\kappa$ B) in the cell cytoplasm. Production of ROS/RNS as well as other stimuli results in phosphorylation of I $\kappa$ B. As a consequence of this p65, a nuclear localisation signal molecule is released with resultant activation of NF  $\kappa$ B. Activated NF  $\kappa$ B then translocates to the nucleus and stimulates transcription factors resulting in the production of pro-inflammatory cytokines such as IL-1 $\beta$ , IL-6(130), IL-8(131, 132), TNF $\alpha$ (133), SLPI(134), and ICAM 1(135, 136). Transactivation of NF- $\kappa$ B by ROS/RNS is synergised by TNF $\alpha$  and IL1 $\beta$  by inducing loss of I $\kappa$ B from the cell cytoplasm (137, 138).

Cellular expression of p65 can be used as a marker of NF- $\kappa$ B activation. Bronchial epithelial biopsies have demonstrated that p65 expression is increased in smokers with or without COPD compared with healthy non smokers. Furthermore, levels of p65 in these cells correlate with FEV<sub>1</sub>(136).

Several other pathways have been implicated in the production of pro-inflammatory cytokines as a result of oxidative stress. Intracellular antioxidant depletion and ROS production results in AP-1 activation(139). The AP-1 pathway is involved in the production of the pro inflammatory cytokines IL1 $\beta$ , TNF $\alpha$  and IL6 along with stimulating the cyclo-oxygenase 2 and prostaglandin synthase 2 pathways with

subsequent increased production of prostaglandins(140, 141). This results in disruption of the structure of the nucleosome(142) and chromatin remodelling(143). Oxidative stress has also been shown to stimulate the Mitogen activated protein kinase pathways(144).

#### **1.4.7 Antioxidant defence systems**

The human lung copes with oxidative stress through various antioxidant systems(145) present in the major airways, the alveoli, the epithelial lining fluid and within the lung cells. Free radicals, reactive oxygen species, reactive nitrogen species and benzenes interact with these antioxidants as they are inhaled. If the amount of these molecules in the lung exceeds the capacity of the antioxidant defence systems then oxidative stress occurs. This results in direct damage to the lung tissue and production of pro-inflammatory cytokines and subsequent inflammatory cell influx. These effects, if chronic, may contribute to the pathological changes in COPD.

Most of the antioxidant capacity of the lung is extra-cellular, present in the epithelial lining fluid (ELF) of the alveoli(146). The most important antioxidant in the epithelial lining fluid is glutathione(147). High molecular weight glycoproteins are secreted in the major airways as a response to oxidative stress and act as a primary defence mechanism for oxidative stress(148).

Several factors impact on the lungs antioxidant capacity. Many of the antioxidant systems are dependant on dietary intake. Production of several systems can be stimulated by oxidative stress, whilst others can be inactivated by oxidative stress.

Enzymatic	Non-Enzymatic
Glutathione-s-transferase	$\beta$ -carotene
Glutathione Peroxidase	Lactoferrin
Haem Oxygenase	Glutathione
Superoxide Dismutase	Uric Acid
Catalase	$\alpha$ -tocopherol
Thioredoxin	Ascorbic Acid

**Table 1.4     Anti-Oxidant Defence Systems**

Antioxidant defence systems can be divided into enzymatic and non enzymatic (149). Ascorbic acid and  $\alpha$ -tocopherol are two non-enzymatic antioxidants which are present in the ELF(150, 151). Other non-enzymatic systems include uric acid, lactoferrin, glutathione and  $\beta$ -carotene(152). Smoking is associated with reduced  $\beta$ -carotene and vitamin C levels in plasma along with reduced vitamin E levels in lung BAL(153-156). The major source of non enzymatic antioxidants is diet.

The role of dietary antioxidants in the pathogenesis of COPD is unclear. There is some evidence that increasing vitamin C is associated with improved lung function



in smokers, asthmatics and patients with bronchitis(157). In contrast dietary supplementation with  $\beta$  carotene resulted in increased presentation of lung cancer(158).

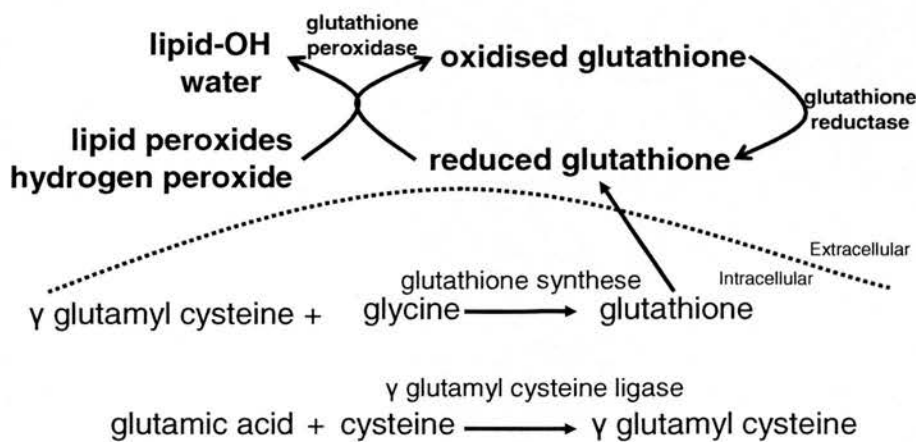
Enzymatic antioxidant systems include superoxide dismutase, catalase, glutathione peroxidase, glutathione-s-transferase and thioredoxin(149). Cigarette smoking leads to increased superoxide dismutase, catalase and glutathione peroxidase activity in the lungs(159).

Superoxide dismutase is present both intra and extra cellularly and directly metabolises superoxide producing hydrogen peroxide and oxygen(149). Three isoforms of superoxide dismutase have been identified(160-162).

Haem Oxygenase (HO-1) is an intracellular enzyme which also exists in three isoforms. The inducible HO, HO-1, is stimulated by oxidative stress(163) resulting in increased production of biliverdin from haem with production of iron and carbon monoxide(164). Bilirubin reductase metabolises biliverdin to form bilirubin, a potent antioxidant(163). Levels of HO-1 are decreased in COPD whilst levels of non inducible HO-2 are increased(101).

As mentioned above, the epithelial lining fluid (ELF) is the major source of antioxidants in the human lungs(146). Glutathione in the ELF acts as a key scavenger for free radicals, hydrogen peroxide and lipid peroxides(165). Glutathione is

synthesised from three peptides, glutamic acid, cysteine and glycine. The two major sources of glutathione intracellularly are the lung and liver(166, 167). In the lung glutathione is produced intra-cellularly and secreted into the ELF. Glutathione can exist in two forms, reduced glutathione (GSH) and oxidised glutathione (GSSG). Various systems exist to maintain glutathione in reduced form in the ELF.



**Figure 1.4.5 Intracellular Production of Glutathione**

The rate of production of glutathione is controlled by three factors; the amount of  $\gamma$  glutamyl cysteine synthase, the amount of cysteine and the amount of glutathione via a negative feedback loop(168). Intracellular glutathione passes into the extracellular epithelial lining fluid by a mechanism which is not fully understood(169). The concentration of glutathione in the lung is 100 times that of plasma, 200-400  $\mu\text{M}$  compared with 2-4 $\mu\text{M}$ . Most cells cannot directly import GSH

and rely on de novo synthesis to maintain intracellular GSH levels(170). The levels of glutathione peroxidase and GSH in lung epithelial lining fluid are elevated in a proportion of smokers compared with non smokers(171, 172).

GSH reacts with hydrogen peroxide in a reaction catalysed by glutathione peroxidase to produce glutathione disulphide (GSSG) and water. Glutathione peroxidase is secreted by epithelial cells and macrophages(173). GSSG is then metabolised to GSH by glutathione reductase,(169, 174). This reaction requires the hexose monophosphate shunt pathway(169). The normal physiological state is to have greater than 90% of glutathione in its reduced form(169). GSH can also interact with superoxide to produce  $GS^{\cdot-}$  and hydrogen peroxide.

Decreased intracellular GSH levels are associated with increases in lung epithelial permeability(119, 172). Cigarette smoke extract induced injury to alveolar epithelial (A549) cells which could be reversed by GSH is enhanced by GSH depletion.(175) The red blood cells of smokers have increased GSH levels compared with non smokers, producing increased protection against hydrogen peroxide induced damage(176).

The enzyme  $\gamma$  Glutamate Cysteine Ligase (previously known as  $\gamma$  Glutamylcysteine synthase) plays a major role in glutathione production (177, 178,179). 4-hydroxy nonenal (4-HNE) is an aldehyde which is a stable end product of lipid peroxidation, and may play a role in regulating gene expression of  $\gamma$

Glutamate Cysteine Ligase in the lung(180). Production of 4-HNE results in increased levels of Transforming Growth Factor  $\beta$ 1 (TGF  $\beta$ 1)(181). TGF  $\beta$ 1 expression correlates with airflow limitation(182, 183). High levels of 4-HNE are associated with increased  $\gamma$ -GCS expression in alveolar epithelial cells(170, 184) via the MAP Kinase pathway. 4-HNE has been shown to be elevated in the resected lungs of patients with COPD when compared with controls(179).

Xanthine Oxidase is a further enzymatic system implicated in oxidative stress in the lung. Activation of this system results in increased generation of superoxide ion and hydrogen peroxide. Xanthine oxidase levels are increased in the lungs of rats exposed to cigarette smoke, associated with increased leukocyte adhesion(185, 186). Furthermore, levels have also been shown to be increased in BAL of patients with COPD compared with healthy non smokers and are associated with increased superoxide generation(187).

In summary, several anti-oxidant systems have been identified which protect against oxidative stress in the lung. The function of these systems appears to play a crucial role in subsequent development of COPD.

#### **1.4.8 Cellular Influx**

Oxidative stress in the lungs results in increased gene expression and production of pro-inflammatory cytokines. This in turn causes both influx and activation of inflammatory leukocytes. Increased numbers of neutrophils, macrophages,

eosinophils and T-cell lymphocytes have all been associated with the development of COPD.

Differential cell counts in induced sputum (IS) show increased neutrophil levels in smokers when compared with non-smokers (187). Bronchial alveolar lavage demonstrates similar results(188). Furthermore, IS neutrophil differentials are higher in smokers with airflow limitation when compared with smokers having normal lung function(184, 189, 190). There is some evidence that the percentage of neutrophils in induced sputum correlates with the rate of decline of FEV<sub>1</sub> in COPD(191).

Higher levels of neutrophils are also seen in the small airways and lung parenchyma in COPD, although this is not as marked as in the larger airways(192). Smokers with COPD have increased number of leucocytes in the small airways of the lung on bronchial biopsy with inflammatory cell numbers correlating with FEV<sub>1</sub>(193). Neutrophilic infiltration of airway smooth muscle has also been shown to correlate with FEV<sub>1</sub>(194).

Neutrophils are thought to play an important role in the pathogenesis of COPD releasing elastase, metalloproteases and free radicals(195). The passage of neutrophils through the pulmonary circulation is dependant on the deformability of these cells as their diameter is greater than that of the average pulmonary capillary(196). Acute smoking results in delay in neutrophil transit in the lungs(197).

Oxidative stress results in increased sequestration of neutrophils by reducing cell deformability(197) an action which is reversed by antioxidants(198).

The pro-inflammatory cytokine IL-8 is a potent chemotaxin for neutrophils and plays a role in recruitment and activation of neutrophil and alveolar macrophages(199). The prostaglandin LTB-4 and the enzyme system xanthine oxidase may also contribute to neutrophil PMN recruitment in the lungs(200, 201).

There is evidence that neutrophils from smokers and patients with COPD have increased chemotaxis and proteolytic activity compared with healthy controls(202). The peripheral neutrophils of smokers with raised levels of neutrophils in the blood produce more superoxide ion than smokers with normal neutrophils counts or non smokers(203). In COPD superoxide generation from neutrophils is increased during acute infections(79).

Neutrophil cellular influx may result in lung blood vessel damage which increases neutrophil- endothelial cell adhesion(204). Neutrophils express a variety of molecules on the cell surface which promotes cellular adhesions. Rabbits exposed to cigarette smoke express higher levels of CD 18 integrins in sequestered neutrophils in pulmonary vessels(186). Neutrophils of patients with chronic bronchitis express more E-selectin compared with healthy controls. Furthermore there is increased expression of intracellular adhesion molecule 1 (ICAM-1) in COPD patients(205). In peripheral blood neutrophils, superoxide production is increased in COPD exacerbations(206).

Although the association between neutrophils and the pathogenesis of COPD is the most frequently studied, other inflammatory cell types have been implicated in the development of COPD.

Alveolar macrophages (AM) also appear to be affected by cigarette smoke and oxidative stress. Macrophages produce pro-inflammatory cytokines and proteases as a result of oxidative stress.

Cigarette smoke appears to influence the morphology of macrophages; AMs in smokers are larger and more pigmented compared with non-smokers(207). When AMs of smokers are stimulated by oxidative stress there is a resultant increase in superoxide production compared with non smokers(208).

Subjects with COPD appear to have higher levels of macrophages; a twenty five fold increase in macrophage numbers is seen in resected lung from COPD patients compared with normal smokers(209, 210). Furthermore, alveolar macrophages in patients with COPD are more active, secreting higher numbers of inflammatory proteins and demonstrating higher elastolytic activity compared with normal smokers(211). There also appears to be an association between macrophage numbers and COPD severity with higher numbers of macrophages in patients with severe compared to mild COPD(184).

Alveolar macrophage numbers are increased in chronic smokers. Smoking cessation has been shown to reduce the numbers of AM and PMN in lung lavage(13, 212). As mentioned above AMs in COPD patients have greater elastolytic activity. The major elastolytic enzyme secreted by AM in patients with COPD is matrix metalloproteinase 9 (MMP-9), whose activity is regulated by the NF $\kappa$ B pathway which itself is strongly associated with the development of COPD(136, 213). Furthermore nuclear staining for p65, a nuclear localisation signal molecule associated with NF- $\kappa$ B, is increased in macrophages during exacerbations of COPD(213, 214)

Together with neutrophils and macrophages, both eosinophils and T-lymphocytes demonstrate associations with COPD. Eosinophils are elevated in airway wall biopsies of patients with COPD(215). There is also increased production of eosinophilic cationic proteins in BAL of patients with COPD(216). Increased T-cell lymphocytes have also been demonstrated in airways and lung tissue of patients



with COPD. These T-cells are predominantly CD8 positive which may contribute to parenchymal destruction(217).

#### **1.4.9 Protease Antiprotease Imbalance**

Chronically increased levels of pro-inflammatory cytokines and inflammatory leukocytes are associated with COPD pathogenesis. One explanation for this is that inflammatory cells produce an excess of protease enzymes such as neutrophil elastase (NE), over antiproteases, such as  $\alpha$ 1Antitrypsin ( $\alpha$ 1-AT). This protease / antiprotease imbalance results in lung tissue damage and subsequent development of COPD.

Excess production of neutrophil elastase results in degradation of elastin, proteoglycans and glycoproteins along with abnormalities in surfactant production(218). NE also induces expression of the cytokine IL-8 in airway epithelial cells(219).

Tracheal instillation of NE in guinea pigs induces emphysema(220). Furthermore NE inhibition reduces cigarette smoke induced emphysema in guinea pigs(221). A proportion of healthy smokers have increased levels of elastase in bronchial lavage (222). There is evidence of increased elastase activity in COPD with immunocytochemical localisation of NE on elastin fibres in emphysema(223).

Further protease enzymes have been identified. MMP-9 is the predominant elastolytic enzyme released by AM in COPD and is regulated by the NF $\kappa$ B pathway(213). Other serine protease include cathepsin G and proteinase 3. The lysosomal cysteine proteases may also play a role in COPD(224).

In parallel with an excess of proteases, development of COPD is associated with inactivation or insufficiency of antiproteases.  $\alpha$ 1-AT is the main antiprotease in lung parenchyma. Subjects with  $\alpha$ 1-AT deficiency develop emphysema early(225). Together with absolute or relative deficiency, inactivation of  $\alpha$ 1-AT has been identified in COPD. Oxidative stress through cigarette smoke, peroxynitrite and chemically generated oxidants has been shown to inactivate antiproteases in vitro(226, 227).

The  $\alpha$ 1-AntiTrypsin protein is susceptible to oxidant damage at its methionine site(222). Increased levels of oxidised  $\alpha$ 1-AT are present in BAL of smokers(228). It appears that  $\alpha$ 1-AT must be inactivated by oxidants for elastase to be toxic in the lung (229, 230). Thus oxidative stress appears to impair antiprotease function, accelerating elastin breakdown(230).

Other antiproteases, such as Secretory Leukoprotease-1 (SLP-1), are present in the airways and may also be inactivated by oxidative stress(231).

#### **1.4.10 Histological Changes in COPD**

The mechanisms described above lead to oxidative stress, inflammation and protease / antiprotease damage to the lung which results in the development of COPD. In keeping with the heterogeneous clinical features of this disease, the histological features of COPD are varied and can include damage to the large and small airways(210) and destruction of the lung parenchyma, resulting in emphysema(232).

The large airways in COPD demonstrate hypertrophy and hyperplasia of the bronchial glands along with goblet cell metaplasia(233). Small airway changes may be detected early in the course of the disease. Small airways have been shown to be narrowed in COPD with increased wall thickness due to collagen deposition and formation of lymphoid follicles. Lumen diameters are also reduced due to inflammatory infiltrates(234).

Emphysema is a result of destruction of alveolar structures(210). Two major forms of emphysema are recognised, centriacinar and panacinar. Centriacinar, also known as centrilobular, relates to damage of alveoli around the respiratory bronchioles and the central portions of the acinus. Centriacinar damage is usually most marked in the upper lobes. Panacinar emphysema is associated with uniform damage of airspaces distal to the terminal bronchioles and predominately affects

the lower lobes(214). Destruction of alveolar walls results in a loss of elastic recoil in the lung(235). Damage impairs perfusion of the lung, resulting in a ventilation / perfusion mismatching(236).

## **1.5 Non-Invasive assessment of Oxidative Stress and Inflammation**

### **1.5.1 Introduction**

A great deal of our understanding of the mechanisms underlying the pathogenesis of Chronic Obstructive Pulmonary Disease is derived from cell and animal studies. Application of these models to the COPD population is problematic. Subjects with COPD are, in general, elderly, have significant respiratory impairment and often have several co-morbidities. As a consequence studies which require invasive sampling, such as from bronchial biopsy, are difficult to perform in significant numbers, with selection bias towards the healthier COPD patients. This has resulted in increasing interest in non-invasive assessment of oxidative stress and inflammation in the COPD population.

Various methods have been identified which may allow measurement of mediators which are associated with COPD pathogenesis. Pulmonary biomarkers may be obtained from the analysis of sputum or through the sampling of cooled exhaled breath, known as exhaled breath condensate. Systemic assessment of biomarkers can be measured from samples of blood or urine.

A large number of studies of non invasive biomarkers in COPD using the methods described above have been published. A selection of these studies is reviewed

below, concentrating specifically on compounds implicated in the oxidative stress hypothesis as the cause of COPD.

As mentioned above, direct quantification of Reactive Oxygen and Reactive Nitrogen Species (ROS / RNS) is problematic due to the short half life of many of these molecules. Several molecules have been identified as biomarkers of oxidative stress. These can be broken into three categories either directly measuring ROS / RNS, measuring stable compounds which are produced when ROS / RNS are metabolised, or measuring the effect of ROS / RNS. Various methods exist to measure the level of oxidative stress, both locally and systemically.

### **1.5.1 Exhaled Breath Condensate (EBC)**

Greater than 99% of exhaled breath condensate is water(237). Non-volatile compounds, both hydrophilic and lipophilic, are also contained within exhaled air. These compounds appear to originate as respiratory droplets in the lung although the precise source of these exhaled compounds is not known(238). Exhaled breath condensate can be collected and concentrations of identified compounds measured. The presence and concentration of these molecules in exhaled breath may be related to disease activity in the lungs.

Various collection systems have been developed, either commercially available or developed within research units, to enable collection of EBC. The underlying principle of these systems is similar. Exhaled breath passes through a one way

collection system and is cooled, usually to  $-20^{\circ}\text{C}$ . As exhaled water vapour cools it condenses and collects in a sterile receptacle. After a set collection time the procedure is terminated and samples either analysed or frozen for subsequent analysis.

Collection and analysis of exhaled breath condensate is a relatively novel method and has several factors which may potentially have a major impact on the concentration of molecules measured. The recent ERS/ATS Task Force on the measurement of EBC identified circadian rhythm, food and drink consumption, medication, co-morbidity, age and gender as potential sources of variability that can effect the measured concentration of various compounds(239).

The current consensus on exhaled breath condensate collection is that subjects should breathe tidally into a one way valve system for a fixed time period. Nose clips reduce nasal contamination and increase sample collection and therefore should be worn. Once collected, breath condensate should be removed from the collection device and frozen at  $-70^{\circ}\text{C}$ . Repeated defrosting of samples may affect analysis; if multiple analyses are planned samples should be aliquoted prior to freezing(239).

Several compounds in exhaled breath condensate have been identified as potential markers of oxidative stress in lung disease. Hydrogen peroxide and 8-Isoprostane have been the most frequently studied and are discussed in detail below.

### 1.5.2 8 Isoprostane

As indicated previously, oxidative stress results in the generation of free radicals. Cell lipid membranes can be damaged by these free radicals, a process which is known as lipid peroxidation(240). F2 $\alpha$ -Isoprostanes are stable end products of lipid peroxidation generated from arachidonic acid and released from cell membranes after this process(241-245). Lipid peroxidation is independent of the cyclooxygenase-1 and cyclooxygenase-2 pathways(242, 246). 8-Isoprostane is one of these stable end products and is thought to be biologically active, possibly as a mediator of cellular inflammation(247, 248).

Previous studies have demonstrated that 8-isoprostane is a reliable marker of lipid peroxidation in vitro(249) and in animal models(245, 250, 251). 8-Isoprostane is measurable in plasma, EBC and urine of healthy individuals(246). 8-Isoprostane levels measured in urine and plasma are increased in several diseases related to controls in a wide range of non respiratory conditions including diabetes(252), severe heart failure(253), ischaemic heart disease(254), Alzheimer's disease(255) and pre-eclampsia(256). Respiratory diseases other than COPD also have elevated levels of 8-Isoprostane compared to controls. This has been demonstrated in both asthma(257) and cystic fibrosis(258).



As a result of its role as a potential marker of oxidative stress 8-Isoprostane has been extensively studied in relationship to smoking, both in healthy individuals and in smoking related lung diseases.

Levels in plasma are increased in healthy smokers compared with healthy non smokers(259). Acute cigarette smoke results in increased 8-Isoprostane levels in exhaled breath condensate both 15 minutes and 5 hours after smoking(260). Lipid peroxidation appears to relate to body mass index (BMI) in smokers, higher BMI being associated with increased 8-Isoprostane. Furthermore, vitamin C supplementation appears to reduce levels of this marker of lipid peroxidation in smokers. This effect is most marked in subjects with a high BMI(261).

8-Isoprostane levels are elevated in the urine of patients with COPD when compared with healthy smoking controls. Increased levels of 8-isoprostane are also seen in the urine and EBC of patients with COPD(262, 263). Urine 8-isoprostane correlates inversely with FEV<sub>1</sub> and PaO<sub>2</sub>(264).

Several papers have studied 8-Isoprostane levels in subjects with COPD in comparison to control subjects. Mean 8-Isoprostane levels are higher in the urine of patients with COPD than healthy smoking controls(265). Furthermore oral antioxidant supplementation results in a decrease in urine 8-isoprostane levels, this decrease correlates inversely with changes in FEV<sub>1</sub> during anti-oxidant supplementation(266). 8-Isoprostane levels in exhaled breath condensate are elevated in subjects with COPD when compared with healthy controls (260).

#### 1.5.4 Hydrogen Peroxide in Exhaled Breath Condensate

Hydrogen peroxide, like 8-Isoprostane, is a product of oxidative stress. Superoxide ion interacts with water to produce this molecule, a process catalysed by superoxide dismutase.  $H_2O_2$  is a stable product and can be measured in exhaled breath condensate.

There is evidence of elevated hydrogen peroxide in a variety of respiratory conditions other than COPD including community acquired pneumonia(267), tuberculosis and sarcoidosis(268), systemic sclerosis(269), mountain sickness(270) and after inhalation of welding fumes(271).

Emelyanov et al demonstrated elevated levels of hydrogen peroxide in EBC of 70 subjects with asthma compared with 17 healthy controls. Furthermore hydrogen peroxide levels appeared to correlate with  $FEV_1$  and bronchial hyperactivity(272). Loukides et al were able to demonstrate a similar pattern in bronchiectasis with elevated hydrogen peroxide levels in 37 patients compared with 25 age matched controls and evidence of correlation between hydrogen peroxide levels and measurement of airflow limitation(273).

Dekhuijzen et al demonstrated elevated  $H_2O_2$  levels in 12 patients with stable COPD compared with 10 healthy controls. The levels were further elevated during exacerbations of COPD(90). Two conflicting trials have recently been published regarding acute changes in hydrogen peroxide levels in EBC during exacerbations

of COPD. Gerritsen et al demonstrated that treatment with high dose systemic corticosteroid reduced levels of EBC hydrogen peroxide during the first 7 days of acute exacerbations(274), whilst van Beurden et al did not demonstrate any significant change in hydrogen peroxide levels in the first week after hospitalisation due to lower respiratory tract infection in subjects with COPD(275). There is some concern about the reproducibility of hydrogen peroxide measurement in EBC, both COPD patients and healthy controls show diurnal variation and day to day variation without change in symptoms, the coefficient of variation of hydrogen peroxide over 21 days in COPD patients is 45%(276).

The effect of inhaled corticosteroid treatment on exhaled breath hydrogen peroxide levels in COPD patients has also been studied. Van Beurden et al demonstrated a decrease in hydrogen peroxide levels in 41 patients with COPD after four weeks treatment with both inhaled fluticasone propionate and beclomethasone dipropionate with no significant difference between effects of the two drugs(277). In contrast Ferreira et al did not demonstrate any significant change in levels of exhaled  $H_2O_2$  after two weeks treatment with inhaled beclomethasone in 20 subjects with COPD(278).

Several papers have examined the effect of antioxidant therapy on exhaled hydrogen peroxide levels. Twelve months of 600mg od N-acetyl cysteine reduced the levels of exhaled hydrogen peroxide at 9 months and 12 months completion in 22 patients with COPD(279). A subsequent study by De Benedetti et al supported this initial study with levels of exhaled hydrogen peroxide falling after 15, 30 and 60 days of 600mg bd N-acetyl cysteine(280).

Administration of nebulised N-acetyl cysteine to 8 healthy subjects resulted in a significant drop in levels of exhaled hydrogen peroxide 30 minutes after treatment. However 3 hours after therapy levels were higher than baseline(281). A further study of the effect of nebulised N-acetyl cysteine in 19 subjects with COPD demonstrated a transient rise in levels of exhaled  $H_2O_2$  after 30 minutes with levels returning to baseline 90 minutes after administration of therapy(282).

Hydrogen Peroxide, as with 8-Isoprostane, is a measurable product of oxidative stress. In studies of small numbers of patients it has been shown to be elevated in

patients with COPD, possibly rising during exacerbations. Antioxidant therapy may reduce levels of Hydrogen Peroxide.

### 1.5.5 Other Biomarkers of Oxidative Stress

Several other biomarkers have been identified as indicative of oxidative stress when measured both locally and systemically. The results of some of these studies are discussed below.

Assessment of the levels of xanthine oxidase (XO), an enzyme involved in the production of superoxide anion and hydrogen peroxide, can be used as a marker in response to oxidative stress. In BAL, levels of XO are higher in COPD compared with healthy smokers and non-smokers(187, 283).

Nitric Oxide interacts with thiols in the epithelial lining fluid to produce nitrothiols, which have been shown to be elevated in EBC of smokers and of patients with COPD, when compared with healthy controls(284). Nitrotyrosine, a product of nitrosative stress, is elevated in the EBC of patients with COPD and inversely correlates to FEV<sub>1</sub>(97). It is also elevated in sputum leucocytes from COPD patients (106). Inducible Nitric Oxide Synthase (iNOS) is increased in the alveolar walls of patients with severe COPD(101).

As mentioned earlier, free radicals cause lipid peroxidation of phospholipids in cell membranes(285). Measurement of products of lipid peroxidation is an indication of the degree of oxidative stress. Pentane and isopentane, both products of lipid peroxidation, are increased in EBC of smokers when compared with healthy controls(286, 287). Elevated levels of ethane, which is generated through oxidative

stress, are measurable in EBC of patients with COPD and correlates inversely with FEV<sub>1</sub>(288). Products of oxidative stress such as malondialdehyde (MDA), acrolein and n-hexanal are all elevated in the IS and EBC of patients with COPD when compared with controls(289).

Peroxides and aldehydes are other products of lipid peroxidation. These products react with thiobarburic acid(290). An increased level of thiobarburic acid reative substances (TBARS) is seen in the blood and EBC of subjects with COPD(291, 292). The levels of TBARS are increased in sputum and inversely correlate with FEV<sub>1</sub>(210). Increased levels of MDA are present in plasma of subjects with COPD compared with healthy controls(293). TBARS as a marker of oxidative stress should be interpreted with caution(294). Previous studies have demonstrated higher levels of TBARS in COPD compared with control subjects, which is most marked after exercise(294, 295). Furthermore oral n-acetylcysteine has been shown to attenuate the increase in TBARS seen after exercise(296). TBARS results should be interpreted with caution. Previous work has demonstrated that certain chemicals, including malondialdehyde and amino acids, when present in biological fluid can react with thiobarburic acid leading to a falsely high level of TBARS(297, 298).

The coenzyme Q-10 (CoQ-10) can be measured in plasma and exists in oxidised and reduced forms. The ratio of oxidised CoQ-10 increases in response to oxidative stress(299). Increased levels of oxidised CoQ-10 are present in COPD versus healthy controls and are reduced with increasing inhaled oxygen(300). Protein carbonyls are produced from amino acids as a result of oxidative stress(301) and are elevated in plasma of health controls after acute cigarette smoking (302).

As summarised above, there are several biomarkers of oxidative stress which may potentially be utilised in the monitoring of a variety of disease processes, including COPD. Application of these biomarkers to larger studies has not as yet been performed.



### **1.5.6 Previous work on Inhaled Corticosteroid and systemic inflammation oxidative stress**

Previous studies have demonstrated that inhaled corticosteroid (ICS) can have some effect on markers of oxidative stress in COPD. Lower levels of nitrotyrosine and inducible Nitric Oxide Synthase in induced sputum have been measured in subjects with COPD after treatment with ICS(303). COPD patients taking ICS have lower levels of exhaled ethane compared with COPD steroid naïve patients(288). In contrast markers of oxidative stress in the exhaled breath condensate of patients with COPD do not appear to be altered. Steroid treatment has failed to demonstrate any effect on levels of 8-Isoprostane, hydrogen peroxide, LTB-4, PGE2, PGD2 and PGF 2 $\alpha$ (263, 304-307).

### 1.5.7 Induced Sputum

Induced Sputum (IS) is another non-invasive method of monitoring disease activity. The principle of this technique is that inhalation of nebulised hypertonic saline aids sputum expectoration. Induced sputum so obtained can subsequently be analysed for differential cell types and markers of inflammation and oxidative stress.

This technique increases the proportion of subjects who are able to provide sputum for analysis in clinical trials when compared with spontaneous sputum sampling. Furthermore a higher percentage of the cells provided are viable when compared with spontaneous sputum.

The utility of induced sputum as a marker of airways disease was first studied in asthmatic patients, and showed increased eosinophils in asthmatic subjects compared with controls(308). Reproducibility studies indicated that differential cell counts and measurement of cytokines such as interleukin 8 and eosinophilic cationic protein were reliable in asthmatic subjects(309).

Similar studies have been carried out in subjects with COPD. Induced sputum provides more viable cells when compared with spontaneous sputum without affecting the differential cell count or measurement of inflammatory markers(310). Measurement of differential cell counts and soluble markers of inflammation has

been shown to be reproducible in patients with COPD(311). Along with evidence of reproducibility, sputum induction is safe in subjects with airways disease even when FEV<sub>1</sub> is less than 40% predicted(312). Use of hypertonic saline delivered by ultrasonic nebuliser is more successful in terms of production of viable sputum when compared with normal saline. Saline delivered at incremental hypertonicity from 3-5% also increases sputum cell viability(313). Induced sputum obtained by this method can provide sufficient sample for analysis in up to 95% of patients with COPD(314).

Previous studies have demonstrated that neutrophils and macrophages are increased in the airways of subjects with COPD when compared with healthy controls,(184, 315) and that the level of neutrophils are inversely proportional to the FEV<sub>1</sub>(316). A direct relationship has been demonstrated between the proportion of neutrophils in sputum and the degree of chronic bronchitic symptoms(317). Neutrophils have also been associated with clinical progress. Higher levels of neutrophils are associated with more rapid decline in FEV<sub>1</sub> and disease progression(318).

Recruitment of neutrophils into the airways therefore appears to be an important factor in the development of COPD. The cytokines IL-8 and TNF- $\alpha$  are neutrophil chemotactic agents(319), which are elevated in sputum supernatant in COPD(320). Previous work has demonstrated a direct relationship between the levels of IL-8 in sputum supernatant and the neutrophils cell count in both BAL(190) and induced sputum(321, 322). Levels of IL-6 and IL-8 are higher when

measured in stable COPD compared to control subjects. Furthermore patients with a high exacerbation frequency have higher levels of IL-8 and IL-6 when compared to COPD patients with low exacerbation rates. IL-8 is also directly associated with the rate of decline of FEV<sub>1</sub>(318). Levels of IL-6 and IL-8 have also been shown to be elevated in exacerbations of COPD compared to the stable state(323, 324).

An association has been demonstrated between IL-6 and IL-8 levels and molecules implicated in oxidative stress. For example, activation of the NF- $\kappa$ B pathway results in increased gene transcription of IL-6, TNF- $\alpha$  and IL-8.(190, 321, 325) Furthermore IL-8 also stimulates degranulation of neutrophils and production of reactive oxygen species.(326, 327)

IL-1 $\beta$  is another pro-inflammatory cytokine implicated in the development of COPD(328). This cytokine stimulates increased production of both IL-8 and TNF- $\alpha$ (329, 330). Furthermore increased production of IL-1 $\beta$  results in enhanced neutrophil adhesion due to increased expression of the intracellular adhesion molecules ICAM-1(331, 332). Elevated levels of ICAM-1 gene expression in pulmonary fibroblasts are found in subjects with COPD (333).

The cytokine Vascular Endothelial Growth Factor (VEGF) has been shown to act as a cell signalling agent in COPD. Increased levels of VEGF are thought to result in endothelial cell proliferation (334) and reduced levels in endothelial cell apoptosis(335). Recent work has suggested that VEGF has different roles in different phenotypes of COPD. Subjects with emphysematous lungs had lower than control VEGF concentrations in the lungs, which directly correlated with FEV<sub>1</sub>. Subjects with predominately chronic bronchitis phenotype had higher levels of

VEGF in induced sputum than control subjects which inversely correlated with FEV<sub>1</sub>.(336)

Several other induced sputum biomarkers have been studied in COPD.

Myeloperoxidase and neutrophil elastase are protease enzymes which are elevated in patients with COPD(337). Matrix metalloproteinase 9 is also elevated in induced sputum of patients with COPD. This protein is associated with extracellular matrix remodelling associated with both COPD and asthma.(338)

In conclusion, induced sputum is a safe, reproducible method which allows non-invasive measurement of differential cells counts and a variety of proteins which may aid our understanding of COPD.

### 1.5.8 Biomarkers of Systemic Inflammation

COPD is not only associated with mortality and morbidity due to lung disease. Relationships also exist between COPD and osteoporosis(339), low BMI(340), skeletal muscle dysfunction(341) and cardiovascular disease(342). These relationships may relate to increased systemic inflammation in COPD(343). There is therefore increasing interest in biomarkers of systemic inflammation.

Several pro-inflammatory cytokines are elevated in plasma of subjects COPD including IL-6 and IL-8(344). Plasma levels of  $\text{TNF}\alpha$  are elevated in COPD subjects with a low BMI compared with those with a normal BMI, and also healthy controls.(345)

Acute phase proteins, such as Fibrinogen and C Reactive Protein (CRP) are produced in the liver as a result of increased inflammation. A recent systematic review of 14 original articles demonstrated that COPD is associated with elevated levels of both CRP and Fibrinogen in serum when compared with healthy controls independent of smoking history(346).

CRP appears to be closely associated with disease activity in COPD. Man et al demonstrated a linear relationship between CRP and  $\text{FEV}_1$ , smoking history, BMI

and blood pressure in 4,803 subjects with mild to moderate COPD. Furthermore elevated CRP levels were associated with increased all cause, cardiovascular and cancer mortality in these subjects over a five year period. CRP did not appear to relate to respiratory mortality(347). A similar pattern is seen in patients with severe COPD, with elevated CRP levels associated with reduced exercise capacity and health status(348).

In conclusion there is evidence of increased systemic inflammation in subjects with COPD. Systemic inflammation appears to relate to disease activity and mortality in this disease.

## 1.6 Anti-Oxidant Treatment Review

Glutathione, as mentioned above, has a significant role in the pathogenesis of COPD. Theoretically administration of glutathione in patients with COPD may be of benefit. However this compound has poor bioavailability when given by mouth limiting its use as a disease modifying agent. One of the major rate limiting factors in the production of glutathione is the intracellular concentration of L-cysteine. This chemical also has a low bioavailability. N-acetylcysteine (NAC) is an immediate precursor of L-cysteine and in contrast is biochemically active in humans. The half life of n-acetyl cysteine is 2.5 hours when administered orally(349) and 5.7 hours when given intravenously(350).

In view of the bioavailability of N-acetyl cysteine and its role as a precursor of glutathione the effect of NAC on oxidative stress and inflammation has been extensively investigated.

In-vitro studies have examined the direct effect of NAC on reactive oxygen species along with the role of NAC in modifying response to oxidative stress in a variety of cell lines. Animal models have also researched the effect of oral administration of NAC and subsequent reactions to oxidative stress.



Studies in human populations have studied the effect of oral NAC on biomarkers of oxidative stress and inflammation in healthy subjects and patients with COPD. Several studies have been performed in clinical practice looking at the effect of oral NAC on endpoints such as exacerbation rates, breathlessness scores and lung function. These studies have included retrospective analysis of the effect of oral NAC along with prospective interventional studies. Finally, several qualitative database reviews exist of these interventional studies. A review of a selection of these studies follows.

The main interventional role of NAC is to increase the level of glutathione in the lungs. Studies have demonstrated this action in human alveolar A549 cells(351), animal models(352) and in human populations, both healthy subjects(128) and those with COPD(353). Along with increasing intracellular cysteine and subsequently glutathione, there is evidence that n-acetyl cysteine may act as a direct scavenger of free radicals. The free thiol group of this molecule can interact with the electrophilic groups present in reactive oxygen species and protects human bronchial fibroblasts against the toxic effects of tobacco smoke condensate in vivo(354). When NAC interacts with ROS, NAC is metabolised to NAC disulphide(355), (356). This process appears to have a mucolytic action in humans when inhaled by breaking down the disulphide bridges of macroproteins(356). In a cell free assay, NAC reduces hydrogen peroxide levels(351). In cellular models NAC has been shown to penetrate red blood cell membranes more effectively than glutathione and improves thiol levels in those cells(357).

NAC also blocks lipopolysaccharide induced apoptosis and maintains glutathione levels in cultured porcine endothelial cells(358). Human polymorphonuclear leukocytes preincubated with NAC in high doses had attenuated response to induced oxidative stress (359). Furthermore NAC inhibits IL-1 $\beta$  induced production of ROS (8-Isoprostane) and monocyte chemotactic protein (MCP-1) and p38 mitogen-activated protein kinases in human airway smooth muscle cells(360).

As indicated above NF  $\kappa$ B activation is a major factor in the gene expression of pro-inflammatory cytokines after oxidative stress. Several studies have demonstrated the effect of NAC on NF  $\kappa$ B activation. Silica induced NF  $\kappa$ B cleavage in human bronchial epithelial cells is inhibited by NAC(361). In vitro NAC treatment of fetal membranes results in a reduction in NF  $\kappa$ B induced production of PGF2 $\alpha$ , IL-6, IL-8, TNF $\alpha$  and 8-Isoprostane after stimulation by lipopolysaccharide(362). High dose in vitro NAC inhibits TNF $\alpha$  induced NF  $\kappa$ B activation of 'monocyte-like' U937 histiocytic lymphoma cells(361). NAC leads to increased GSH levels both in absolute amounts and in relation to GSSG in endothelial cells. Initial rises in GSH were associated with an increase in iNOS gene stimulation. However at higher levels of GSH iNOS gene expression was inhibited(363).

In an animal model, cigarette smoke inhalation induced increased airway epithelium thickness and increased numbers of secretory cells in pathogen free rats. Administration of oral NAC inhibited both of these effects. It should be noted that NAC doses were very high in this study with an average daily dose of

973mg/kg, compared with human studies of between 200mg and 1800mg per patient per day(364). Administration of NAC after smoking cessation reduced the time to recovery of secretory cell numbers(365). A further study examined the protective effects of oral administration of NAC in rats prior to instillation of elastase. This demonstrated that administration of NAC for two days attenuated elastase induced emphysema. Assessment of induced emphysema was by measurement of collagen content, alveolar surface area and airflow(366). Oral administration of NAC to mice did not affect the levels of NAC measured in the lung indicating poor bioavailability. However tobacco smoke induced glutathione depletion in the lungs of mice was prevented by administration of NAC as were levels of hydrogen peroxide production(354).

Two doses of NAC are commonly studied in humans, NAC 600mg three times a day (high dose), and 600mg once a day (low dose). The short term effect of NAC on glutathione levels has previously been studied. Administration of low dose (600mg od) of NAC has been shown to result in increased levels of glutathione in plasma and bronchial lavage of healthy subjects after 5 days administration(128). Plasma levels of glutathione are not increased in patients with COPD after low dose NAC. However high dose (600mg tds) has been shown to increase plasma glutathione in COPD. Neither dosage of NAC has any influence on BAL levels of glutathione in patients with COPD(353).

8 weeks of oral NAC (200mg tds) in smokers did not affect cell numbers in BAL however the proportion of lymphocytes increased to levels comparable to non smoking controls. The phagocytic capacity of Alveolar Macrophages improved after administration of NAC in 5 of the 11 smokers studied and the ability of AMs to respond to oxidative stress improved(367).

Long term treatment with oral NAC (200mg tds) in healthy smokers resulted in reduced levels of lactoferrin (LF), anti-chymotrypsin and chemotactic activity of neutrophils in BAL. In plasma from the same individuals, concentrations of myeloperoxidase and elastase were reduced(368). Low dose NAC (600mg od) in patients with COPD over a twelve month period resulted in reduced levels of hydrogen peroxide in exhaled breath condensate after 9 and 12 months of treatment, but not after 6 months, versus placebo. This medication did not have any impact on levels of TBARS in plasma(369).

Gerrits et al performed a retrospective analysis studying the impact of the introduction of NAC at first hospital presentation of exacerbation of COPD on readmission with exacerbations in the first year of treatment. This demonstrated that NAC appeared to reduce the risk of readmission by 33% after adjustment for disease severity. This reduction appeared to be dose related. Although small numbers received more than 200mg od of NAC (19 of the 97 patients studied) relative risk for readmission was 0.55 compared with the group who did not receive NAC (370).

In contrast, acute administration of low dose NAC (600mg od) along with conventional therapy at the time of presentation with an exacerbation of COPD did not affect recovery from exacerbation in terms of length of stay, improvement in lung function, breathlessness scores or oxygen saturations(371).

Although several interventional studies with NAC in patients with either COPD or chronic bronchitis have been performed, individual results from these trials have been inconclusive. Most recently the Bronchus trial, a large multicentre, double blind placebo control trial of 600mg od of NAC over 3 years versus placebo, was performed in 523 patients. No affect was noted on exacerbation rates or rate of decline in FEV<sub>1</sub>. Sub group analysis of steroid naïve patients demonstrated a relative risk reduction for exacerbation of 0.79, however no effect was demonstrated on spirometry in this group (372).

A Cochrane database review of randomised, double blind, placebo controlled studies with a variety of oral mucolytics, including n acetyl cysteine, was performed. Trials included in the review were to be of at least two months therapy in subjects with chronic bronchitis (21 papers) or COPD (2 papers). This demonstrated a reduction in exacerbations of COPD compared with placebo. Six

trial subjects required treatment with an oral mucolytic to prevent one exacerbation in the study period (range 2 months to 24 months) compared with placebo(373).

A quantitative systematic literature review of the use of NAC specifically has also been carried out. This review identified 11 randomised placebo controlled trials of NAC, daily doses between 400mg and 600mg, in treatment of chronic bronchitis over periods between 12 and 24 months. Nine of the 11 papers used exacerbation rate as an end point. Overall a reduction in exacerbations was identified in NAC versus placebo (30-60% vs 40-81%), with 5.8 patients requiring treatment for trial duration to prevent one exacerbation. An improvement in symptoms of breathlessness was also noted in the 5 trials which used this as an endpoint, 61.4% demonstrating an improvement in SOB score in NAC versus 34.6% in placebo(374).

In conclusion oral NAC appears to play a role in the management of COPD. This is most likely due to increasing intracellular reduced glutathione although the chemical itself may also have direct anti-oxidant effect along with mucolytic properties. Administration of NAC has demonstrated evidence of reduced oxidative stress along with attenuated response to various agents related to oxidative stress. However the response in clinical studies of oral NAC has been variable.

This variable response demonstrated in clinical studies may relate to the limited bioavailability of N-acetyl-cysteine. As mentioned above, low dose NAC had no

effect on glutathione in BAL. NAC is acidic, thus limiting its usefulness as inhaled therapy due to bronchospasm and febrile reactions. This has led to the development of Nacystelyn (NAL), the lysine salt of N-acetylcysteine ( $C_{11}H_{23}N_3O_5S$ ). Nacystelyn has a neutral pH, and is well tolerated when given by inhalers in clinical studies in healthy adult smokers at doses up to 80 mg bd over 4.5 days(375, 376). Inhaled N-acetylcysteine or nacystelyn therapy may more effectively improve antioxidant capacity in the lungs compared with oral treatment.

When the efficacy of NAL was studied in a cell free assay both NAC and NAL were shown to inhibit hydrogen peroxide, but not superoxide, action comparable to glutathione. Cultured A549 cells incubated with NAC results in an increase in levels of total glutathione. This increase is significantly greater (two fold) after culture with NAL. NAC and NAL have both been shown to reduce hydrogen peroxide release from PMN cells of smokers with COPD by around 45%. Activation of phagocytic cells by serum induced zymosan and subsequent ROS generation as measured by luminal enhanced chemiluminescence is inhibited by 50% by NAL at lower concentrations than either NAC or captopril(377). The pH of NAC is 3.6 and of NAL 6.4(378).

When nacystelyn comes into contact with mucus fluid it dissociates into the two base compounds, lysine and N-acetylcysteine. These compounds are then either incorporated into the host cells or excreted without accumulation or retention(379-382). Administration of NAL using a mono dose inhaler and gelatine capsules has

an in-vivo lung deposition of 30% nominal dose in healthy subjects(383) and 23.5% in adults with CF(384).

Previous studies have been performed examining the role of Nacystelyn in human lung disease. Subjects with cystic fibrosis were administered NAL in a phase II, multicentre, clinical trial(385). This demonstrated a dose dependent reduction in the primary study rheological endpoint,  $\log G^*$ , a measure of sputum viscosity, at all time points. This improvement in sputum rheology appeared to correlate with clinical improvement as reflected in a reduction in the number of respiratory infections, the increase in time to first respiratory infection, and a reduction in the number of days of absence from work or school.



## 1.7 Conclusion

Chronic Obstructive Pulmonary Disease is a common condition which is increasing in prevalence and has a major impact in terms of morbidity and mortality on individuals and society.

COPD is strongly, but not exclusively, associated with cigarette smoking. Several other factors, including diet, socioeconomic status and air pollution affect the development of this disease. There is evidence that COPD pathogenesis is associated with increased levels of oxidative stress in the lungs, leading to inflammation and tissue damage. Treatment with anti-oxidant therapy may play a role in the management of COPD.

Several methods of monitoring non-invasive biomarkers of inflammation and oxidative stress have been identified which may be of use in furthering our understanding of the pathogenesis of this disease.

## 1.8 Hypothesis and Aims

*The hypotheses of this thesis are as follows:*

- Oxidative stress and subsequent airways inflammation are major factors in the pathogenesis of COPD;
- Non invasive biomarkers of oxidative stress and airways inflammation can be measured in the exhaled breath condensate and induced sputum of subjects with COPD and controls;
- Measurement of these biomarkers is reproducible during three visits in a four week period;
- The level of oxidative stress and inflammation measured with these biomarkers relates to severity of disease;
- Treatment with inhaled anti-oxidant reduces the level of oxidative stress and inflammation resulting in reduced levels of biomarkers and improved health status.

*The aims of this thesis were as follows:*

- Identify which biomarkers can be measured in induced sputum and exhaled breath condensate;
- Assess the reproducibility of these biomarkers;
- Relate levels of inflammation and oxidative stress to severity of COPD;
- Assess the response to an inhaled antioxidant in subjects with COPD.

## **Chapter 2**

### **Materials and Methods**

## Chapter 2 Materials and Methods

The study protocols, method of statistical analysis and ethical approval for each individual study are discussed at the start of the appropriate chapter.

## 2.1 Standard Operating Procedures for sample collection

### 2.11 Exhaled Breath Condensate Collection

Exhaled Breath Condensate was collected using a Jaeger EcoScreen device. The mouthpiece, collection port, collection tube, saliva trap and breathing valve were sterilised between each individual use.

Exhaled carbon monoxide was assessed immediately prior to the test to exclude acute smoking. Subjects were asked to rinse their mouth with water prior to the test and to wear nose clips. They were then asked to perform normal tidal breathing for 10 minutes.

If there was a build up of saliva during collection, subjects were asked to stop the test and remove this. Subjects were allowed to stop collection during the test for up to one minute.

Once collection was completed samples were stored on ice and placed in a -80-°C freezer within 30 minutes of collection. Samples were stored as 0.25ml aliquots.  
(Appendix 2.1)

### 2.1.2 Induced Sputum Collection

Induced sputum was obtained from subjects after baseline FEV<sub>1</sub> post bronchodilator was measured. Those subjects whose FEV<sub>1</sub> was less than 1 litre were reviewed by a member of medical staff prior to continuing with the procedure.

Subjects were asked to rinse their mouth and expectorate any spontaneous sputum which was then discarded. Hypertonic saline (5mls of 3% saline) was then inhaled via a Sonix 2000 nebuliser. After nebulisation subjects were asked to clear their nasal passages, rinse their mouth then attempt expectoration. Sputum obtained was inspected macroscopically and, if felt to be suitable, the procedure stopped.

If no suitable sputum was obtained, spirometry was repeated. If there was a less than 10% fall in FEV<sub>1</sub> from baseline, 4% nebulised saline was given. If there was a fall between 10% and 20% in FEV<sub>1</sub> from baseline, then hypertonic saline at the same concentration (3%) was administered. If a fall greater than 20% from baseline FEV<sub>1</sub> was demonstrated the procedure was abandoned and nebulised bronchodilator administered.

If no suitable sputum was obtained with 4% saline, the procedure was repeated with 5% saline.

In all subjects at the end of the procedure FEV<sub>1</sub> was checked. Nebulised bronchodilator was given if a greater than 10% fall from baseline FEV<sub>1</sub> was noted, and subjects remained in the unit until FEV<sub>1</sub> had returned to >90% baseline.

(Appendix 2.2)

### 2.1.3 Induced Sputum Processing

All sputum samples were processed within 2 hours of production by the method of Popov et al(386). In summary, sputum plugs were separated from contaminating saliva by macroscopic examination, and the weight measured.

A freshly prepared solution of 0.1% dithiothreitol (DTT: from a stock 1% in sterile distilled water) in phosphate buffered saline (PBS), which was 4 times the weight of the selected sputum was added, vortexed for 30 seconds and mixed on ice for 15 minutes to homogenise the mucus. A volume of PBS equivalent to the DTT volume was then added, and the sample filtered through 48  $\mu$ m nylon gauze to remove mucus and debris, but not the cells.

The cells were centrifuged at 1200 rpm for 10 minutes at 4°C. The supernatant was decanted, centrifuged at 2500 rpm for 8 minutes at 4°C, and stored in aliquots at -70°C. The cells were washed with PBS, and centrifuged at 1000 rpm for 8 minutes at 4°C. The total cell count, viability (exclusion of trypan blue), and percentage squamous cell contamination was assessed using a Neubauer haemocytometer. Cytospins were made in a Shandon Cytospin 3 by centrifugation at 300rpm for 3 minutes. (Appendix 2.3)

#### 2.1.4 Blood and Urine Sampling

Blood samples were taken after exhaled breath condensate collection.

Venesection was performed using a Vacutainer system. Plasma was collected in a 9ml Lithium Heparin and a 5ml EDTA tube whilst 5ml of serum was obtained in an anti-coagulant free tube.

Once plasma was collected it was centrifuged then aliquoted. Plasma was stored at  $-80^{\circ}\text{C}$  until analysis was performed. Serum samples were allowed to clot at room temperature for twenty minutes then centrifuged, aliquoted and stored at  $-80^{\circ}\text{C}$ .

Urine was collected in a Sterilin tube and stored at  $-80^{\circ}\text{C}$ . (Appendix 2.4)



### 2.1.5 Spirometric Assessment

Spirometry was measured using a Vitalograph spirometer which was calibrated prior to use.

FEV<sub>1</sub> and FVC were measured first. Subjects were asked to take a full inspiration then exhaled as hard, as fast and as long as possible into the Spirometer mouthpiece. At least three measurements were calculated. A satisfactory result was obtained if there was less than 5% variation between the best test and two other results.

Slow Vital Capacity was also calculated. Subjects were asked to complete a full, steady, non-forced exhalation after a full inspiration. The higher of the two vital capacities measured, Forced Vital Capacity or Slow Vital Capacity was taken as the accurate measure.

Reversibility to bronchodilator was then assessed. Patients were given 2.5mg nebulised salbutamol. Lung function was repeated between 15 and 30 minutes after nebulisation. (Appendix 2.5)

## **2.2 Protocols for Sample Measurement**

### **2.2.1 Exhaled Breath Condensate Assays**

#### **2.2.1.1 8-Isoprostane and Leukotriene B4 measurement**

8-Isoprostane was measured in exhaled breath condensate using a commercially available enzyme immunoassay kit from Cayman Chemicals, AnnArbour, US . In brief, the sample or standard was added to a coated well along with anti-serum specific for 8-Isoprostane and 8-Isoprostane linked to tracer. The plate was then incubated at room temperature overnight. Free 8-isoprostane and 8-Isoprostane linked to tracer completely bind to the anti-serum. The anti-serum binds to the mouse monoclonal antibody which is coated on the well.

The following day, after 18 hours incubation, wells were washed and Ellman's reagent added to the wells. This reagent reacts with the tracer producing a yellow colour. The intensity of this colour is measured spectrophotometrically at a wavelength of between 405 and 420nm. The intensity of colour is proportional to the amount of 8-Isoprostane linked to tracer and therefore inversely proportional to the amount of free 8-Isoprostane.

Standard curves were plotted and concentration of 8-Isoprostane in samples calculated.

Leukotriene B4 was measured using a similar kit also available from Cayman Chemicals. (Appendix 2.6)

### 2.2.1.2 Hydrogen Peroxide Assay

Hydrogen peroxide was measured spectrophotometrically using the method devised by Gallati and Pracht. In brief horseradish peroxidase was added to tetramethylbenzidine dissolved in citrate buffer at a pH of 3.8. 110 $\mu$ L of this solution was added to 100 $\mu$ L of sample or standard in a sterile 96 well plate. These two solutions react producing a blue colour. The concentration of hydrogen peroxide was related to the colour intensity. The reaction was terminated using sulphuric acid after 20 minutes resulting in the fluid in the wells changing to yellow. The intensity of this colour was measured spectrophotometrically at 450nm. Standard curves were then plotted and concentration of hydrogen peroxide in samples calculated. (Appendix 2.7)

### **2.2.1.3 Nitrotyrosine Assay**

Nitrotyrosine levels in exhaled breath condensate was calculated using a commercially available kit from Assay Designs Chemicals.

In brief, sample or standards were added to precoated wells. Wells were incubated for one hour then washed and nitrotyrosine antibody added and incubated for one hour. Plates were then washed and streptavidin peroxidase added. After a further hour plates, were washed and TMB substrate added to the wells and incubated for 30 minutes in darkness. The reaction was stopped by sulphuric acid and intensity of yellow colour measured spectrophotometrically at 450nm.

Standard curves were plotted and sample concentration calculated. (Appendix 2.8)

## 2.2.2 Induced Sputum Assays

### 2.2.2.1 Cytokine measurement in Induced Sputum Supernatant

The cytokines IL-1 $\beta$ , IL-6, IL-8, TNF- $\alpha$  and VEGF were all measured using a commercially available kit from R+D Chemicals. Kits were supplied with capture antibody and detection antibody specific for the cytokine to be measured.

To summarise, sterile 96 well plates were incubated overnight with capture antibody. The wells were then washed and blocked for one hour. Samples and standard were then added to the wells and incubated for two hours. The appropriate cytokine would bind to the capture antibody. Plates were then washed and detection antibody added to bind to this complex. Plates were incubated for a further two hours. Streptavidin horseradish peroxidase, which binds to detection antibody, was then added to the sample, incubated and washed. TMB was added to the wells producing a reaction which was stopped using sulphuric acid after 30 minutes.

The sample solution is yellow in colour, the intensity of yellow being dependant on the amount of streptavidin-HRP in the wells which in turn reflects the concentration of cytokine in the well. This was measured spectrophotometrically at 450nm, standard curve plotted and sample concentration calculated. (Appendix 2.9)

## **2.2.3 Blood Sampling Assays**

### **2.2.3.1 C-Reactive Protein Measurement**

High sensitivity C-Reactive Protein was measured using a commercially available ELISA kit from ImmunDiagnostiks.

In brief, CRP standard or samples were added to wells precoated with polyclonal capture antibodies to CRP and incubated for one hour. Plates were then washed and CRP antibody, labeled with peroxidase, added and incubated. Plates were then washed again and TMB substrate added which reacts with peroxidase. This reaction was stopped with sulphuric acid and the intensity of yellow colour measured spectrophotometrically at 450nm. A standard curve was then plotted and sample concentration calculated. (Appendix 2.10)

### 2.2.3.2 Thiobarbituric Acid Reactive Substance Measurement

Thiobarbituric Acid Reactive Substances were measured in plasma based on the method of Conti et al(387).

In brief, TCA was added to plasma then incubated at 95°C for 30 minutes. Supernatant was removed and Thiobarbituric Acid added to the samples. Tetramethoxypropane was used as a standard. Sodium Dodecyl Sulphate and acetic acid were also added to this solution. Samples solutions were incubated for 60 minutes at 95°C. TBA reacts with aldehydes and lipid peroxides, known as Thiobarbituric Acid Reactive Substances (TBARS), producing a pink colour. The intensity of this colour is dependant on the concentration of TBARS. N-butanol was added to the solution, centrifuged and the organic layer removed. The intensity of colour was read flurometrically at 515nm, standard curve plotted and sample concentration calculated. (Appendix 2.11)

## **Chapter 3**

### **Reproducibility of Biomarkers of Inflammation and Oxidative Stress Measured in Exhaled Breath Condensate and Induced Sputum in Subjects with COPD and Controls**



## Chapter 3

### Reproducibility of Biomarkers of Inflammation and Oxidative Stress Measured in Exhaled Breath Condensate and Induced Sputum in Subjects with COPD and Controls

#### 3.1 Introduction

The pathogenesis of Chronic Obstructive Pulmonary Disease is not yet fully understood. As mentioned in Chapter 1, oxidative stress leading to pulmonary and systemic inflammation may play a significant role in the development of this disease. Non-invasive measurement of molecules which relate to this process may aid our understanding of the disease. Several published papers have demonstrated significant differences in cross sectional studies when the levels of a variety of biomarkers in both exhaled breath condensate and induced sputum of subjects with COPD are compared with healthy controls. These data are discussed in further detail in Chapter 1.

COPD is a heterogeneous disease with a large variation in the level of disability and in the rate of progression of the disease process. Longitudinal monitoring of biomarkers of oxidative stress and inflammation may help identify subjects with aggressive disease who may benefit from intensive treatment. Furthermore, measurement of biomarkers may be of use in assessing response to treatment in interventional studies.

Interpretation of data from longitudinal or interventional studies is dependant on the reliability of the biomarkers measured. There is little information in the published literature on the reproducibility of non-invasive biomarkers. This chapter describes two methodology studies to assess the variability of biomarkers in induced sputum and exhaled breath condensate.

## **3.2 Reproducibility of Differential Cell Counts, Total Cell Counts and Inflammatory Cytokines in Induced Sputum in subjects with COPD and controls.**

### **3.2.1.1 Study Population**

Subjects were recruited from local COPD clinics, GP practices and by local advertisement. Subjects were asked to give written informed consent to the trial prior to participation.

COPD subjects were required to have a documented diagnosis of COPD, had to be over 40 years of age and be either a current or ex-smoker (defined as no tobacco consumption for 6 months) with a minimum of 10 pack years tobacco exposure. One pack year was defined as the equivalent of 20 cigarettes per day for one year. A clinical diagnosis of COPD had to be supported by spirometric evidence of airflow limitation, with post bronchodilator FEV<sub>1</sub> less than 80% predicted and with FEV<sub>1</sub>/FVC ratio less than 70%. Subjects with significant improvement after bronchodilator, defined as greater than 12% improvement in FEV<sub>1</sub>, or complete reversal of airflow limitation were excluded from the trial.

Study visits were only performed on clinically stable patients, defined as no exacerbation in the last month or no exacerbation requiring hospital admission in the last 2 months. Subjects who had experienced more than two exacerbations of COPD in the last 6 months were excluded from the trial. An exacerbation was defined as a change in baseline dyspnoea, cough and / or sputum production sufficient to merit a change in management. (Appendix 3.1.1-3). Symptoms of cough, sputum production and wheeze were scored as either zero (no symptoms), 1 (symptoms present but not constant) or 2 (symptoms present throughout day)

Control subjects were recruited from local GP practices and by local advertising. Subjects were over the age of 40. Current and Ex-Smokers were required to have a smoking history of at least 10 pack years. Ex-Smokers must have abstained for at least 6 months. Non-smokers had to be either lifelong non-smokers or have a smoking history of less than 1 pack year. All subjects were to be free from any clinically significant documented disease which the study doctor felt may either compromise the subject's safety during the trial or interfere with the study results. Controls were required to have normal lung volumes on spirometry, defined as both FEV<sub>1</sub> and FVC greater than 80 percent predicted with no significant reversibility and FEV<sub>1</sub>/FVC ratio greater than 70%. (Appendix 3.1)

A number of exclusion criteria were used in this study and applied to both COPD patients and healthy controls. These included current or previous history of atopy, sinusitis, respiratory disease other than COPD, unexplained respiratory symptoms, any recent upper or lower respiratory tract infection and non-respiratory inflammatory disease. (Appendix 3.1) Subjects with significant symptoms of peptic ulcer disease or gastro-oesophageal reflux were excluded from the trial.

### 3.2.1.2 Study Visits

Prior to study attendance, subjects were asked to abstain from smoking and caffeine for a minimum of 8 hours. Inhaled corticosteroids and bronchodilators were withheld on the morning of clinic attendance. Trial participation was deferred if subjects had received any antibiotics within the last 4 weeks or oral corticosteroids within the last 8 weeks. Subjects who reported significant variation in their respiratory symptoms over the past 4 weeks had trial entry deferred.

Subjects who demonstrated significant between-visit variation in post bronchodilator FEV<sub>1</sub>, defined as out with the range of 90-115% of initial FEV<sub>1</sub>, were excluded from the trial.

On the day of the study, after informed consent to participate in the trial had been obtained, clinical history was reviewed to ensure suitability for trial participation.

Past medical history, drug history, occupational history and family history was recorded. Smoking history was noted including the time of the last cigarette.

Height, weight, body mass index, blood pressure, oxygen saturation and pulse were measured. Exhaled carbon monoxide level was measured and exhaled breath condensate collection was undertaken if there was no evidence of acute cigarette smoke exposure.

Lung spirometry was then measured using a standardised procedure (Appendix 2.5). Induced sputum was then obtained, analysed and supernatant stored as per the study protocol (Appendix 2.2,2.3). Sputum specimens were frozen at -80 °C and analyzed in batches using standard assay protocols (Appendix 2.9).

After the initial study visit, subjects were asked to return in a further 14 ( $\pm 3$ ) days at the same time of day, within one hour, of previous clinic attendance. On the second study visit, a brief medical review was performed to ensure no significant change in respiratory symptoms over the last two weeks and spirometry was repeated to ensure post bronchodilator FEV<sub>1</sub> remained within 90-115% of the previous measurement. Induced sputum samples were then collected. A further study visit was then arranged 14 ( $\pm 3$ ) days from visit 2. The same protocol was followed at visit 3.

### **3.2.1.3 Statistical Analysis**

Analysis between groups was performed using Tukey One Way Analysis of Variance (ANOVA). Contingency tables were analyzed using Fisher's Exact Test. Intra subject variability was assessed using the concordance correlation coefficient. This coefficient quantifies the precision and accuracy of paired observations. One set of measurements are plotted on the y axis and the other on the x axis. The concordance correlation coefficient relates how close the plotted line is to 45°. A value of 1.00 is equivalent to a group of paired observations being exactly the same(388). Intra subject variability was plotted using a Bland Altman Plot of mean level between measurements versus difference between measurements where 95% of plots should lie within the 95% confidence intervals ( $\pm 1.96 \times \text{SD}$ ).

### **3.2.1.4 Ethics Approval**

Ethical approval for this study was obtained from the ethics committee of Lothian Acute Hospitals NHS Trust. Prior to trial participation, subjects were issued with written information regarding the study and given a minimum of 24 hours to digest

this information. Written informed consent was given and signed prior to trial recruitment.

## 3.2.2 Study Details and Demographics

### 3.2.2.1 Study Details

A total of 57 subjects were recruited for the reproducibility study. Twenty seven of the subjects had COPD and 30 were controls. Twelve of the COPD subjects were current smokers. Six of the controls were current smokers, 12 ex-smokers and 12 non-smokers.

Forty seven of the 57 patients completed the trial. Twenty one subjects with COPD, eight of whom were current smokers, and 26 control subjects, 4 smokers, 10 ex-smokers and 12 non-smokers completed. Eight subjects withdrew as a result of respiratory illness, one through non-respiratory illness and one withdrawal was for social reasons.

The demographics of the trial population are discussed below. These data describe the subjects who completed the trial.

### 3.2.2.2 Demographics

The mean age of subjects who were lifelong non-smokers without respiratory disease was lower ( $52.1 \text{ years} \pm 7.8$ ) than smokers with COPD ( $69.9 \text{ years} \pm 5.6$ ,  $p < 0.001$ ), ex-smokers with COPD ( $72.2 \text{ years} \pm 7.8$ ,  $p < 0.001$ ) and control ex-smokers ( $63.2 \text{ years} \pm 9.4$ ,  $p < 0.05$ ). No other significant differences in mean ages were noted between the groups, table 3.1.

There was no significant difference in smoking pack years between the four groups with a smoking history. There was no significant variation in the gender, body mass index or level of co-morbidity between the 5 study groups, table 3.1.



Smokers and ex-smokers with COPD had significantly lower FEV<sub>1</sub>, FVC and FEV<sub>1</sub>/FVC ratio compared with the three control groups. There was no significant difference in spirometric parameters between the two COPD groups or between the three healthy control groups, table 3.2.

There was no significant variation in terms of therapy, health status, exacerbation frequency or symptom scores between the two COPD groups, tables 3.3, 3.4.

Group	COPD Smoker	COPD Ex	Healthy Smoker	Healthy Ex	Healthy Non
Number	8	13	4	10	12
Age mean±stdev	69.9±5.6	72.2±7.8	61.3±13.9	63.2±9.4	52.1±7.8
Sex (m/f)	3/5	10/3	3/1	6/4	5/7
BMI Mean±stdev	24.2±2.8	27.2±6.6	26.2±3.8	26.7±4.3	24.7±2.8
Pack Years Mean±stdev	48.5±27	48.6±24	40±18	24.7±9	0.4±1.4
IHD	0	3	1	0	0
HBP	1	2	0	3	0
Diabetes	0	1	0	0	0

**Table 3.1 Patient Demographics**

BMI= Body mass index, FFM= Fat Free Mass, IHD= Ischaemic Heart Disease,

HBP= High Blood Pressure

Group	COPD Smoker	COPD Ex	Healthy Smoker	Healthy Ex	Healthy Non
FEV <sub>1</sub> Post BD (L) Mean ± stdev	1.2 ± 0.3	1.2 ± 0.4	2.6 ± 0.3	3.0 ± 0.6	3.2 ± 0.6
FVC Post BD (L) Mean ± stdev	2.3 ± 0.9	2.5 ± 0.8	3.7 ± 0.3	4.0 ± 0.8	4.1 ± 0.9
FEV <sub>1</sub> /FVC Ratio Mean ± stdev	57 ± 16	47 ± 12	71 ± 6	78 ± 7	80 ± 7
FEV <sub>1</sub> Post BD (%Pred) Mean ± stdev	52.7 ± 12	46.5 ± 15	89 ± 6	110 ± 12	106 ± 12
%change post BD Mean ± stdev	7 ± 6	11 ± 11	4 ± 6	3 ± 3	1 ± 3
Vol.Change postBD Mean ± stdev	99±87	111±101	95±152	84±107	36±89

Table 3.2 Spirometry Results per Study Group

FEV<sub>1</sub>=Forced Expiratory Volume in 1 sec, FVC=Forced Vital Capacity,

BD=bronchodilator

Group	COPD Smoker	COPD Ex Smoker
Number	8	13
MRC Chronic Bronchitis Mean $\pm$ St Dev	0.9 $\pm$ 0.4	0.5 $\pm$ 0.5
MRC Dyspnoea Mean $\pm$ St Dev	3.0 $\pm$ 1.1	3.4 $\pm$ 1.0
Cough (0-2) Mean $\pm$ St Dev	1.4 $\pm$ 0.7	1.1 $\pm$ 1.0
Sputum (0-2) Mean $\pm$ St Dev	1.9 $\pm$ 0.4	1.2 $\pm$ 0.8
Wheeze (0-2) Mean $\pm$ St Dev	1.1 $\pm$ 0.8	0.7 $\pm$ 0.6
Exac Freq Mean $\pm$ St Dev	1.8 $\pm$ 1.3	1.4 $\pm$ 1.5

Table 3.3 Health Status Results per study group

Group	COPD Smoker	COPD Ex Smoker
Number	8	13
SGRQ Symptoms Mean $\pm$ St Dev	68.7 $\pm$ 17.8	60.4 $\pm$ 21.9
SGRQ Activity Mean $\pm$ St Dev	64.5 $\pm$ 21.4	77.3 $\pm$ 18.5
SGRQ Impact Mean $\pm$ St Dev	36.0 $\pm$ 22.0	40.3 $\pm$ 16.6
SGRQ Total Mean $\pm$ St Dev	50.4 $\pm$ 18.6	55.1 $\pm$ 15.5

Table 3.4 St Georges Respiratory Questionnaire in Current and Ex-Smokers with COPD

Group	COPD Smoker	COPD Ex Smoker
Number	8	13
SABA	4	9
LABA	0	5
SAAC	1	6
LAAC	3	3
ICS	2	7
Theophylline	0	0

**Table 3.5 COPD Treatment in Current and Ex Smoking Groups**

SABA= short acting  $\beta$  agonist, LABA= long acting  $\beta$  agonist, SAAC= short acting anti-cholinergic, LAAC= long acting anti-cholinergic, ICS= Inhaled Corticosteroid

### 3.2.3 Reproducibility of Biomarkers in Induced Sputum

#### 3.2.3.1 Sputum Production in Trial Completers

Forty seven subjects completed the trial. Twenty three of these subjects (49%) were able to provide sufficient sputum for analysis on all three visits. A further six patients (13%) were able to provide sputum on two of the three study visits. Three subjects (6%) provided sputum on one of the visits whilst 15 (32%) were unable to provide sputum on any of the study visits. Table 3.6

There was a significant difference in the ability to provide sufficient sputum for analysis between subjects with COPD and non smoking controls. Eighteen of the 21 (86%) subjects with COPD were able to provide sputum for analysis on all three study visits whilst the other three subjects (14%) provided sputum on two of the three visits.

In contrast only 2 of the 10 ex-smokers (20%) and none of the 13 lifelong non-smokers provided sputum on all three study visits. Three ex-smokers (30%) provided sputum on one or two of the study visits and five (50%) failed to provide sputum on all three visits. Three non-smokers (23%) provided one or two sputum samples, 10 non-smokers (77%) were unable to produce induced sputum during the trial. The three control smokers who completed the trial were all able to provide sputum on the three study visits. Statistical analysis confirms that subjects with COPD were more likely to provide sputum on at least two visits when compared with ex or non smoking controls ( $p < 0.001$ ). Table 3.6. Reproducibility analysis was performed on all subjects who provided sufficient sputum for analysis.

Group	COPD Smoker	COPD Ex	Healthy Smoker	Healthy Ex	Healthy Non
No Sputum	0	0	0	5	10
Sputum on 1 visit	0	0	0	1	2
Sputum on 2 visits	2	1	0	2	1
Sputum on 3 visits	6	12	3	2	0

**Table 3.6 Sputum Production during trial per study group**

Subjects with COPD were more likely to produce sputum on at least two visits (21 of 21) during the trial when compared with control non and ex-smokers (5 of 23),  $p<0.001$ .



### **3.2.3.2 Reproducibility of Differential Cell Counts**

#### **3.2.3.2.1 Reproducibility of Total Cell Counts**

Total cell counts in induced sputum did not vary significantly between the five groups. The reproducibility of total cell count in study subjects is expressed as the concordance correlation. Overall the concordance of total cell counts in induced sputum between visits was 0.65,  $p=0.0002$  (95% confidence interval 0.35 to 0.85).

Figure 3.1

#### **3.2.3.2.2 Reproducibility of Differential Neutrophil Counts**

Subjects with COPD had a higher mean percentage neutrophils in the sputum when compared with controls,  $88.9\% \pm 11.2\%$  versus  $76.4\% \pm 13.3\%$ ,  $p=0.026$ .

The overall concordance correlation coefficient of differential neutrophil cell counts was 0.26,  $p=0.21$  (95% confidence interval -0.14 to 0.60). Separate analysis of the COPD population demonstrated improved reproducibility in this group, with a concordance correlation of 0.45 although this did not reach statistical significance,  $p=0.09$  (95% confidence interval 0 to 0.75). Figure 3.2

#### **3.2.3.2.3 Reproducibility of Differential Macrophage Counts**

Subjects with COPD had a significantly lower mean percentage of macrophages in sputum ( $7.9\% \pm 6.9\%$ ) compared with control subjects ( $22.0\% \pm 12.7\%$ ),  $p=0.0015$ .

The concordance correlation coefficient for this group was 0.58,  $p=0.0007$  (95% confidence interval 0.29 to 0.78). Figure 3.3

#### 3.2.3.2.4 Reproducibility of Differential Eosinophil Counts

There was no significant difference between the percentage of eosinophils in the differential counts in the COPD population compared with healthy controls.

Furthermore serial differential counts did not correlate well with a concordance coefficient 0.19  $p=0.4$  (95% confidence interval -0.22 to 0.54). Figure 3.4

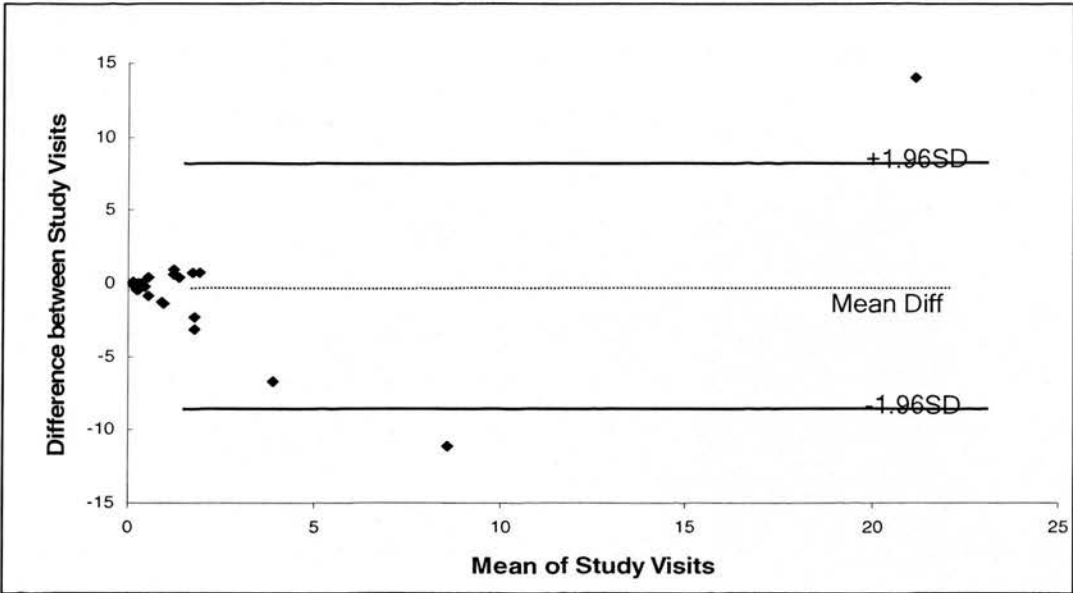


Figure 3.1 Bland Altman Plot Total Cell Counts

Dot plots represent the mean of study visits for each individual subject in the x axis against the difference between study visits in the y axis. The dashed line represents the bias between visits (-0.4 cells per ml) and the solid lines the limit of agreement of the test, -8.4 to 7.6 cells per ml.

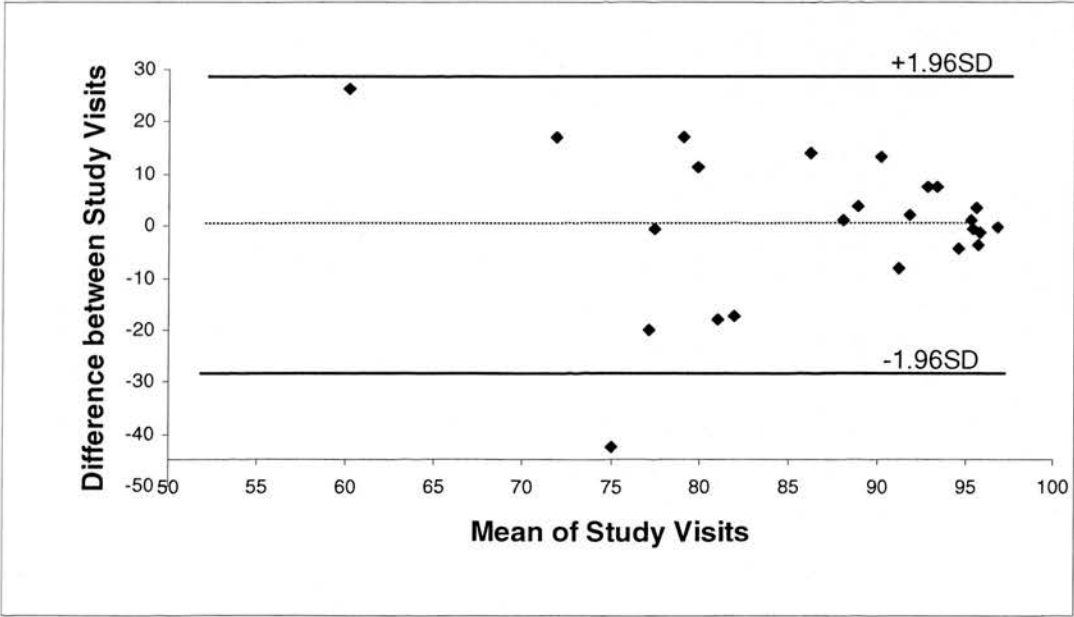


Figure 3.2 Bland Altman Plot Differential Neutrophil Cell Counts

Dot plots represent the mean of study visits for each individual subject in the x axis against the difference between study visits in the y axis. The dashed line represents the bias between visits (0.3% neutrophils) and the solid lines the limit of agreement of the test, -28.1% to 28.8%

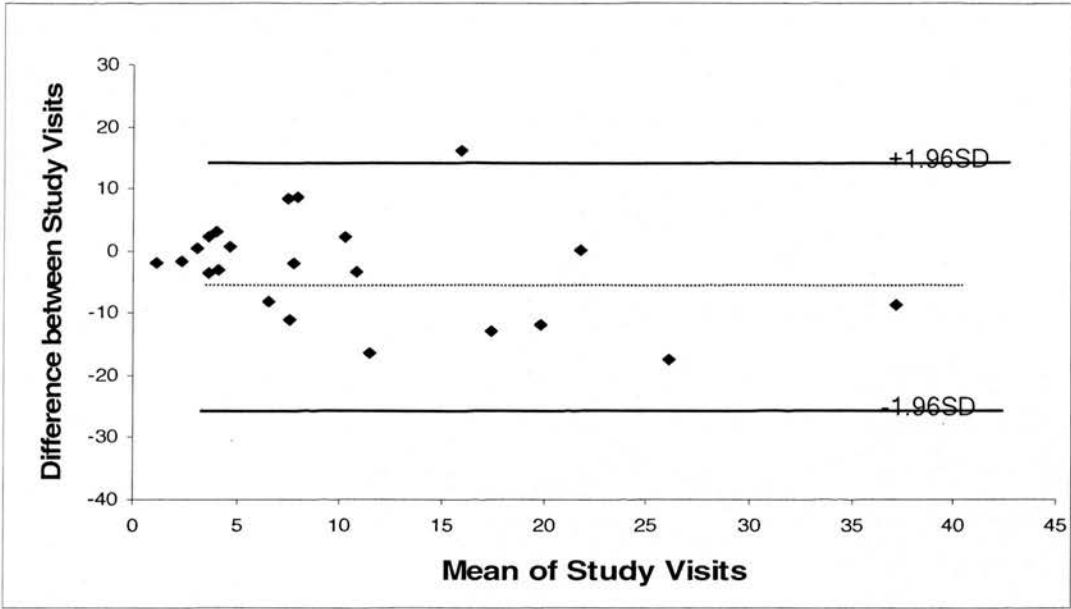


Figure 3.3 Bland Altman Plot Differential Macrophage Cell Counts

Dot plots represent the mean of study visits for each individual subject in the x axis against the difference between study visits in the y axis. The dashed line represents the bias between visits (-2.9% macrophages) and the solid lines the limit of agreement of the test, -23.2% to 17.5%..

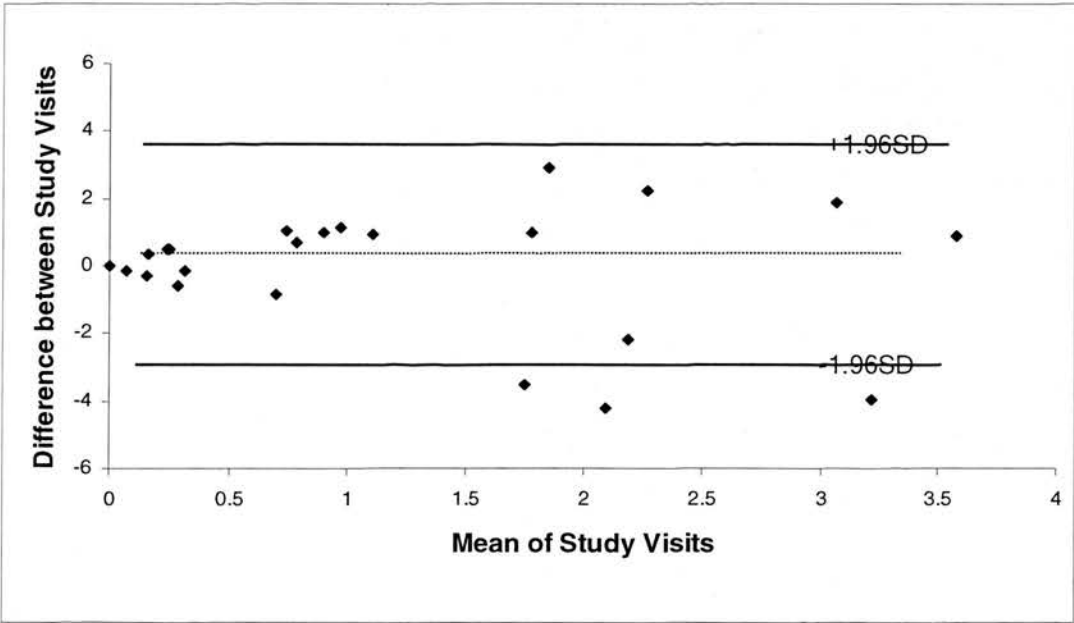


Figure 3.4 Bland Altman Plot Differential Eosinophil Cell Counts

Dot plots represent the mean of study visits for each individual subject in the x axis against the difference between study visits in the y axis. The dashed line represents the bias between visits (0% eosinophils) and the solid lines the limit of agreement of the test, -3.5 to 3.5 % eosinophils.

### **3.2.3.2 Reproducibility of Cytokines in Induced Sputum Supernatant**

#### **3.2.3.3.1 Reproducibility of IL-1 $\beta$**

Levels of IL-1 $\beta$  were measured in the sputum supernatant of the 29 subjects who provided induced sputum. Only 19 of these samples were within the detection limit of the assay, with 9 samples being lower than the limit of detection of the assay (4pg/ml). The level of IL-1 $\beta$  in the one remaining sample was above the limit of detection of the assay (500pg/ml), insufficient sputum supernatant was available to repeat this assay after diluting the sample.

No significant differences were noted in the levels of IL-1 $\beta$  measured in induced sputum between the COPD and control groups. Concordance correlation coefficient between study visits was 0.06,  $p=0.08$  (95% confidence interval -0.3 to 0.4). Figure 3.5

#### **3.2.3.3.2 Reproducibility of IL-6**

Levels of the cytokine IL-6 were measured between the limits of detection of the assay in 22 of the 29 samples obtained, with 7 samples being below the limit of detection. There was no significant difference in the levels of IL-6 measured between the COPD and control groups.

Concordance correlation coefficient between study visits was 0.24 ,  $p=0.30$  (95% confidence interval -0.22 to 0.62). Figure 3.6

#### **3.2.3.3.3 Reproducibility of IL-8**

IL-8 was detected in 25 of the 29 sputum samples obtained on visit 1. Four samples were greater than the limit of detection for the assay ( $>1000\text{pg/ml}$ ). There was no significant difference in levels of IL-8 in the COPD and control groups.

Concordance correlation was 0.43,  $p=0.04$  between study visits with 95% confidence interval 0.02 to 0.7. Figure 3.7

#### **3.2.3.3.4 Reproducibility of VEGF**

VEGF levels were detected in twenty five of the 29 subjects in the trial with four samples below the limit of detection of the assay ( $<15\text{pg/ml}$ ). There was no significant difference between the control and COPD groups.

Concordance correlation was 0.67 between study visits,  $p<0.001$  (95% confidence interval 0.34 to 0.84). Figure 3.8

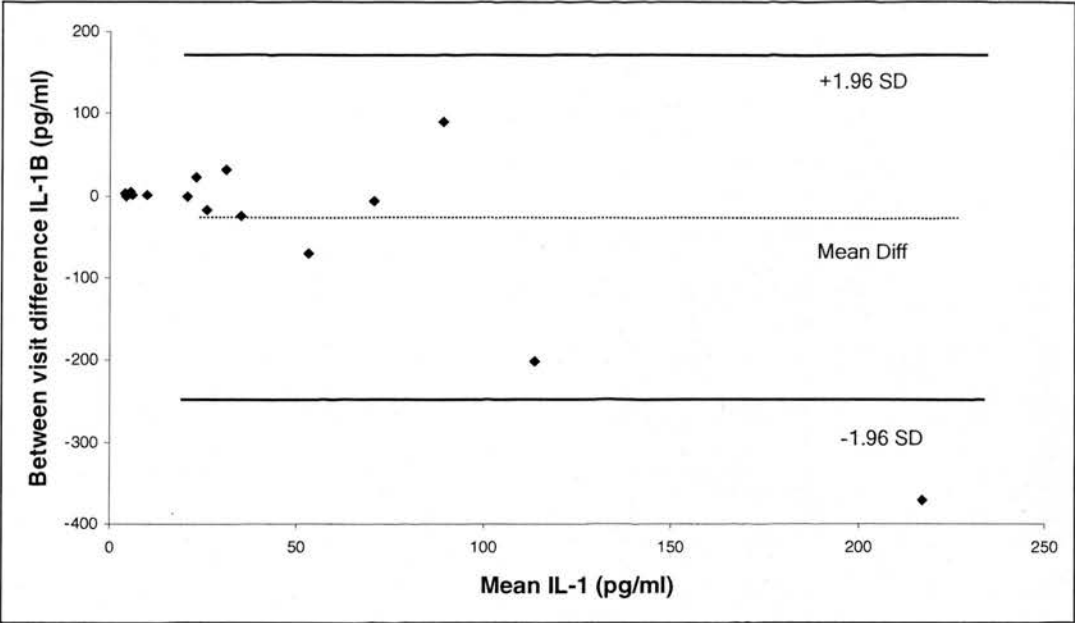


Figure 3.5 Bland Altman Plot IL-1 $\beta$  measurement in Induced Sputum

Dot plots represent the mean of study visits for each individual subject in the x axis against the difference between study visits in the y axis. The dashed line represents the bias between visits (-35 pg/ml) and the solid lines the limit of agreement of the test, -254 pg/ml to 183 pg/ml.

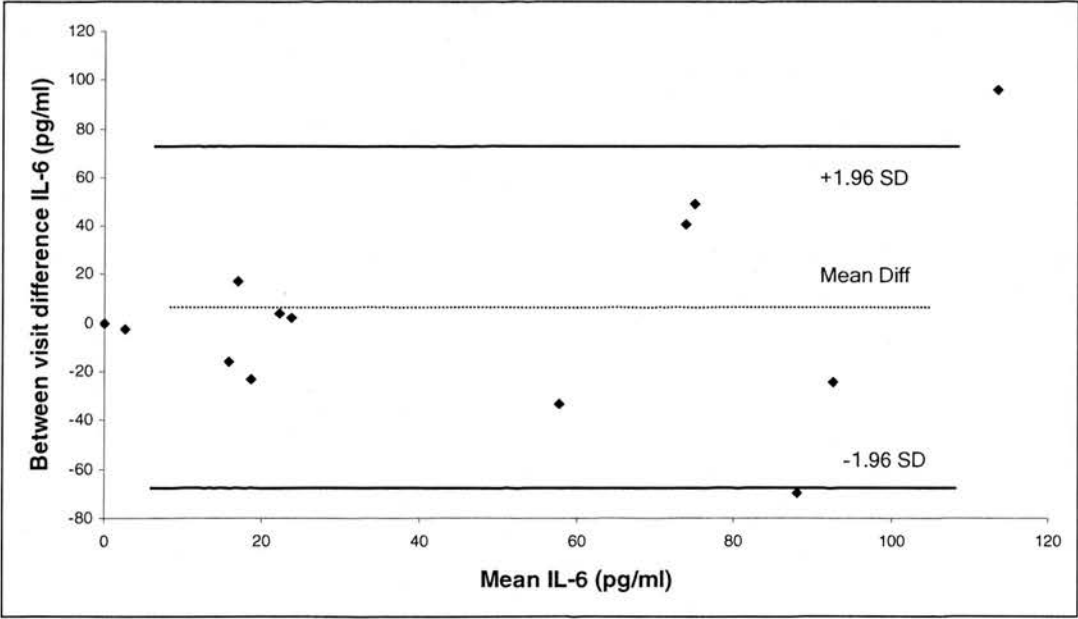
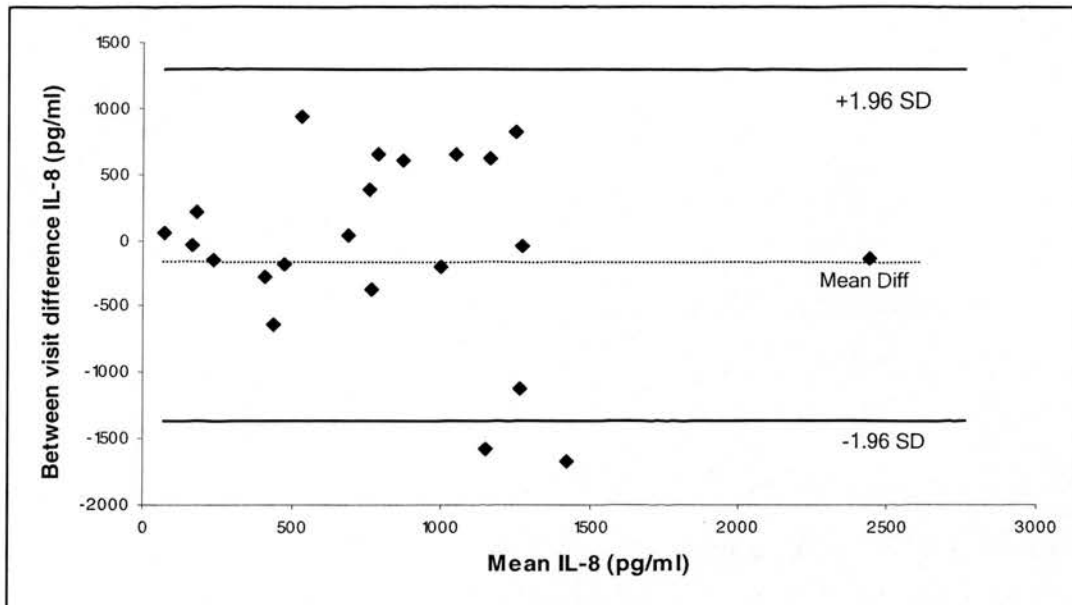
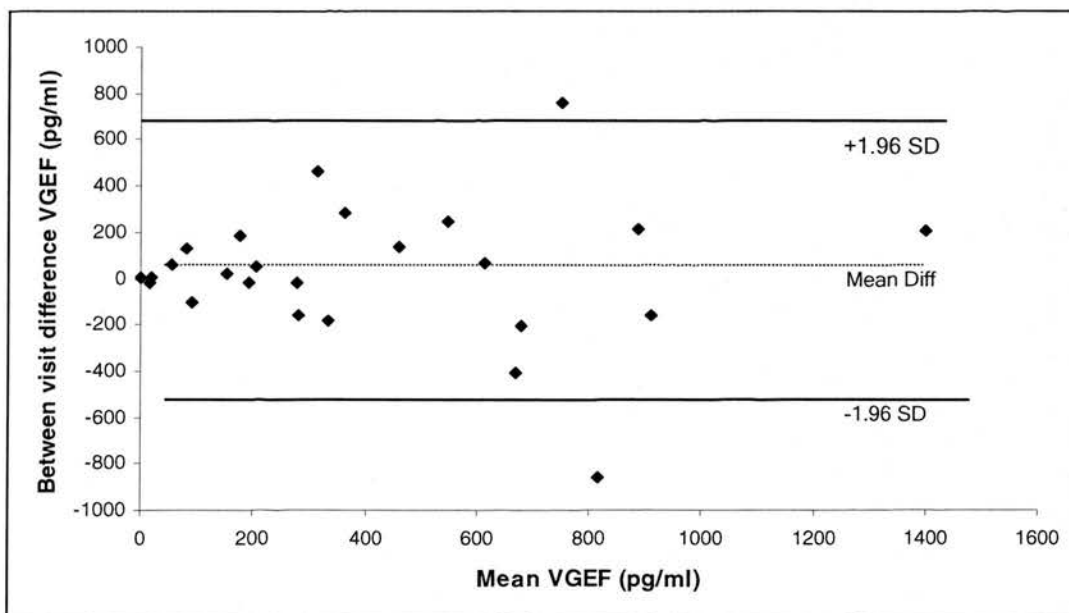


Figure 3.6 Bland Altman Plot IL-6 measurement in Induced Sputum

Dot plots represent the mean of study visits for each individual subject in the x axis against the difference between study visits in the y axis. The dashed line represents the bias between visits (3pg/ml) and the solid lines the limit of agreement of the test, -72pg/ml to 78pg/ml



**Figure 3.7 Bland Altman Plot IL-8 measurement in Induced Sputum** Dot plots represent the mean of study visits for each individual subject in the x axis against the difference between study visits in the y axis. The dashed line represents the bias between visits (-50 pg/ml). and the solid lines the limit of agreement of the test, -1440 pg/ml to 1340 pg/ml.



**Figure 3.8 Bland Altman Plot VEGF measurement in Induced Sputum**

Dot plots represent the mean of study visits for each individual subject in the x axis against the difference between study visits in the y axis. The dashed line represents the bias between visits (25 pg/ml) and the solid lines the limit of agreement of the test, -560pg/ml to 610 pg/ml.



### 3.2.3 Conclusion

Obtaining induced sputum for analysis in subjects without COPD was problematic, particularly in control non or ex-smokers. Interpretation of the reproducibility of induced sputum in this population is therefore not possible.

Sputum induction was more successful in the COPD population with all subjects able to provide sputum on at least two occasions.

The total cell counts obtained appeared reproducible in the study population with a concordance coefficient of 0.65 during the trial ( $p < 0.001$ ). The percentage of macrophages in the differential cell counts also correlated (0.58) between subjects during the trial,  $p < 0.001$ .

In contrast the percentage of neutrophils and eosinophils counted in sputum did not closely correlate between subject visits, although neutrophils differential counts were close to significance in the COPD population ( $p = 0.09$ ).

The cytokines IL-1 $\beta$  and IL-6 were not reproducible in the supernatant of induced sputum. In contrast measurements of levels of IL-8 (correlation coefficient 0.43,  $p = 0.04$ ) and VEGF (correlation coefficient 0.67,  $p < 0.001$ ) in sputum were reproducible during the trial.

### **3.3 Reproducibility of Biomarkers of Oxidative Stress Measured in Exhaled Breath Condensate in Subjects with COPD and Controls**

#### **3.3.1.1 Study Population**

Subjects were recruited from local COPD clinics, GP practices and by local advertisement. Subjects were asked to give written informed consent to the trial prior to participation.

COPD subjects had to meet the same criteria used in the induced sputum trial, again study visits were only performed on clinically stable patients.

Control subjects were recruited by local advertising. Control subjects were not matched for either age or smoking history. All subjects were to be free from any clinically significant disease which was felt may either compromise the subject's safety during the trial or interfere with the study results.

Clinical and spirometric inclusion and exclusion criteria were similar to the induced sputum trial. (appendix 3.1)

### **3.3.1.2 Study Visits**

Study visits followed the same protocol as in the Induced Sputum reproducibility trial mentioned above. Exhaled breath condensate was collected from the subjects using a standardised procedure (Appendix 2.1). Spirometry was then measured using a standardised procedure. EBC specimens were frozen at  $-80^{\circ}\text{C}$  and analyzed in batches using standard assay protocols (appendix 2.6, 2.7).

After the initial study visit subjects were asked to return in a further 14 ( $\pm 3$ ) days at the same time of day (within one hour) of previous clinic attendance. On the second study visit a brief medical review was performed to ensure no significant change in respiratory symptoms over the last two weeks and spirometry was repeated to ensure post-bronchodilator  $\text{FEV}_1$  remained within the 90-115% range. Exhaled breath condensate collection was then repeated.

### **3.3.1.3 Statistical Analysis**

Analysis between groups was performed using Tukey One Way Analysis of Variance (ANOVA). Intra subject variability was assessed using the concordance correlation co-efficient. Intra subject variability was plotted using Bland Altman Plot of mean level between measurements versus difference between measurements.

### **3.3.1.3 Ethical Approval**

Ethical approval for this study was obtained from the ethics committee of Lothian Acute Hospitals NHS Trust. Prior to trial participation, subjects were issued with written information regarding the study and given a minimum of 24 hours to digest this information. Written informed consent was given and signed prior to trial recruitment.

### 3.3.2 Study Details and Demographics of Study Population

A total of twenty three subjects participated in the trial. Five of the subjects were control smokers, five control life long non-smokers and thirteen had Chronic Obstructive Pulmonary Disease.

Twenty one of the subjects completed the trial. One of the COPD subjects had an exacerbation of her disease between visits one and two and one of the healthy non smoking controls discovered that she was pregnant during the trial. Demographic data below discusses the subjects who completed the trial.

The control populations were not age matched with the COPD group. The mean age of the control group was  $29.2 \pm 6.4$  for the control smokers and  $29.8 \pm 5.4$  years for the control non-smokers. The mean age of the COPD group was  $67.7 \pm 6.4$  years. Three of the ten control group were male and ten of the thirteen COPD patients male. Table 3.7

Four of the COPD subjects were current smokers and nine ex-smokers. Average pack years for the control smokers was  $9.5 \pm 5.0$  pack years and  $52.2 \pm 37.1$  for the COPD subjects. The mean St Georges Health Questionnaire Total score was  $57.8 \pm 15.4$  points. Table 3.7

All subjects in the control group had normal lung volumes and  $FEV_1/FVC$  ratio. All COPD subjects had airflow obstruction on spirometry with a mean  $FEV_1$  of  $1.2 \pm 0.7$  litres,  $40.8 \pm 21.7\%$  predicted. Table 3.7

Group	COPD	Healthy Smoker	Healthy Non
Number	13	5	5
Smoking Status (Current/Ex/Non)	4/9/0	5/0/0	0/0/5
Age mean±stdev	67.7±6.4	29.2±6.4	29.8±5.4
Sex (m/f)	10 / 3	1 / 4	2 / 3
Pack Years mean+stdev	52.2±37	9.5±5.0	0
FEV <sub>1</sub> Post BD (L) Mean ± stdev	1.2 ± 0.7	3.3 ± 0.4	3.7 ± 0.7
FEV <sub>1</sub> Post BD (%Pred) Mean ± stdev	40.8 ± 21.7	104 ± 12.2	98.2 ± 10.3
FEV <sub>1</sub> /FVC Ratio Mean ± stdev	46.2 ± 15.4	84.6 ± 2.2	82.1 = 5.8
St Georges Resp Questionnaire Total Score	57.8 ± 15.4		

**Table 3.7 Demographics of COPD and Control Groups EBC Reproducibility Study**

FEV<sub>1</sub>=Forced Expiratory Volume in 1 sec, FVC=Forced Vital Capacity,  
BD=bronchodilator

### 3.3.3 Reproducibility of Biomarkers in Exhaled Breath Condensate

Exhaled breath condensate was obtained on both visits in all 22 subjects. 8-Isoprostane was measured in all 44 samples. Hydrogen peroxide levels were measured on both visits in fourteen of the trial participants, 4 non-smokers, 5 healthy smokers and 5 patients with COPD.

#### 3.3.3.1 Reproducibility of 8-Isoprostane

Levels of 8-Isoprostane were detected in the majority of samples. Two of the twenty two samples on visit one, five of the twenty two on visit two were below the limit of detection for the assay (1.9pg/ml). Levels of 8-Isoprostane measured in the remaining samples were also low with 16 of the remaining 20 samples less than 10pg/ml on visit one, 14 of 17 below 10pg/ml on visit two. There was no significant difference in the levels of 8-isoprostane measured in the three study groups.

The 8-Isoprostane measured did not demonstrate Gaussian distribution when tested using the Kolmogorov and Smirnov method ( $p < 0.05$ ) and was skewed to the left for both visits. Figure 3.9a,b. After data was transformed to the log it had normal distribution, Kolmogorov and Smirnov  $> 0.1$ . Reproducibility analysis was carried out on log transformed data.

Reproducibility of log 8-Isoprostane levels comparing between visits is expressed as the concordance correlation with 95% confidence intervals. There was a just significant correlation between log 8-Isoprostane levels at visit 1 and at visit 2 for the study population as a whole, correlation coefficient 0.28, 95% confidence interval 0.02 to 0.50,  $p = 0.034$ . Figure 3.10

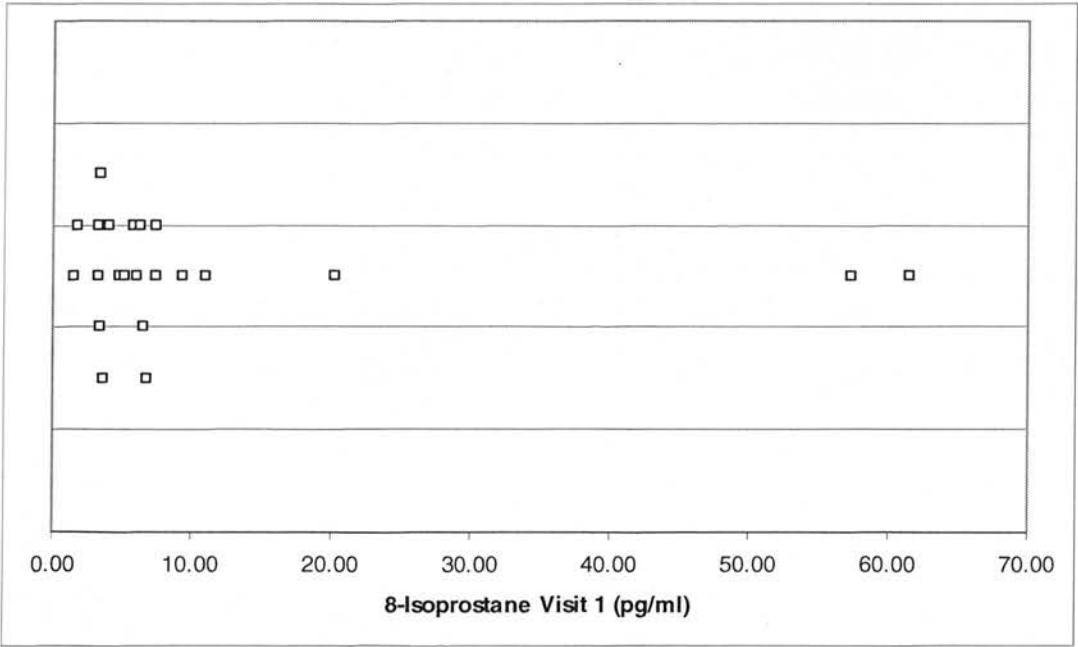


Figure 3.9a

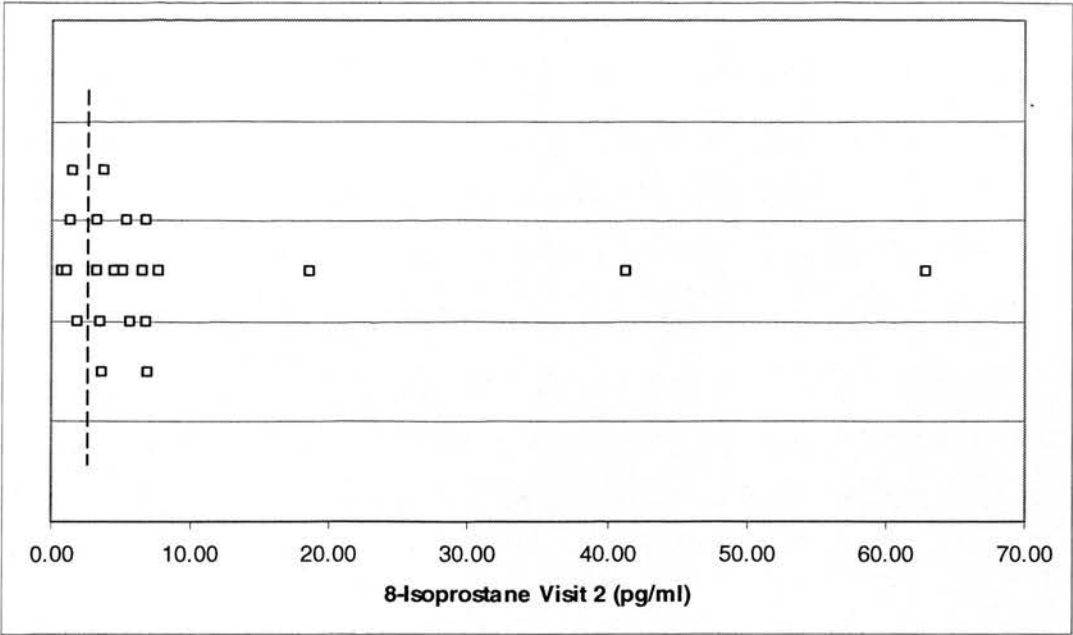


Figure 3.9b

Figure 3.9 Dot Plot Representation of 8-Isoprostane levels measured at Visit 1 (3.9a) and Visit 2 (3.9b)

Plots represent 8-Isoprostane levels per individual for visit 1 and visit 2 of the trial. Dashed vertical line represents Limit of Detection of Assay (1.9 pg./ml)



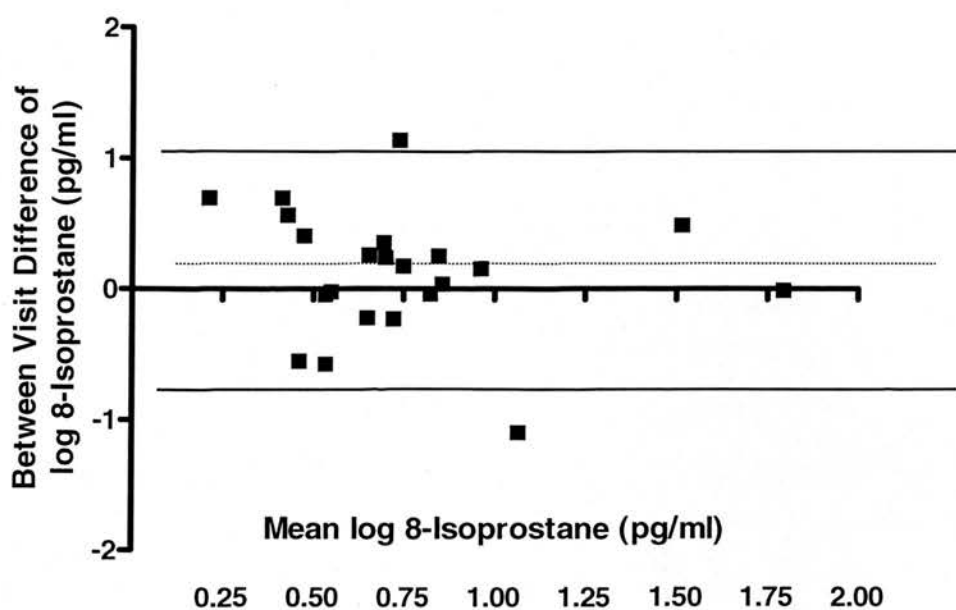


Figure 3.10 Bland Altman plot log 8-Isoprostane levels in Exhaled Breath Condensate- COPD, smokers and non-smokers

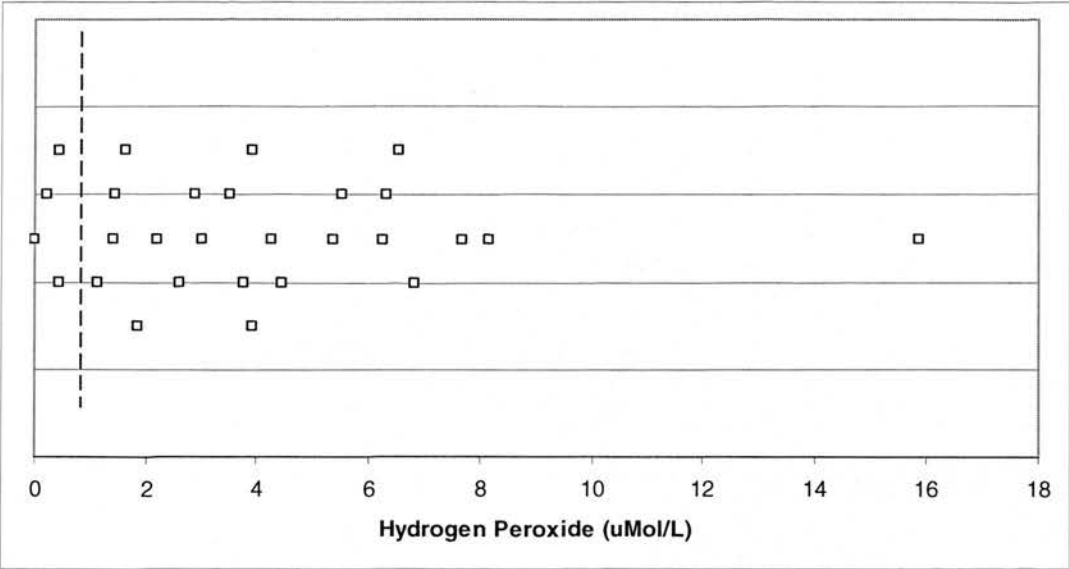
Plots represent the mean log 8-Isoprostane levels of the two visits for individuals compared to the difference between the two measurements. The dashed line represents the bias between visits (0.12 pg/ml) and the solid lines the limit of agreement of the test, -- 0.83 pg/ml to 1.07pg/ml.

### 3.3.3.2 Reproducibility of Hydrogen Peroxide

Hydrogen peroxide levels were measured in exhaled breath condensate (EBC) in 14 individuals on two occasions, five smokers, 4 non-smokers and 5 subjects with COPD. Twelve of the 14 subjects had levels of hydrogen peroxide above the lower limit of detection of the assay (0.78 $\mu$ mol/L) on visit 1. At visit 2 again 12 of the 14 subjects had detectable hydrogen peroxide in EBC, although it was not the same individuals who did not have detectable levels when compared to visit 1. Hydrogen peroxide levels measured in EBC in this study demonstrated Gaussian distribution, Kolmogorov and Smirnov  $p > 0.1$ . Figure 3.12

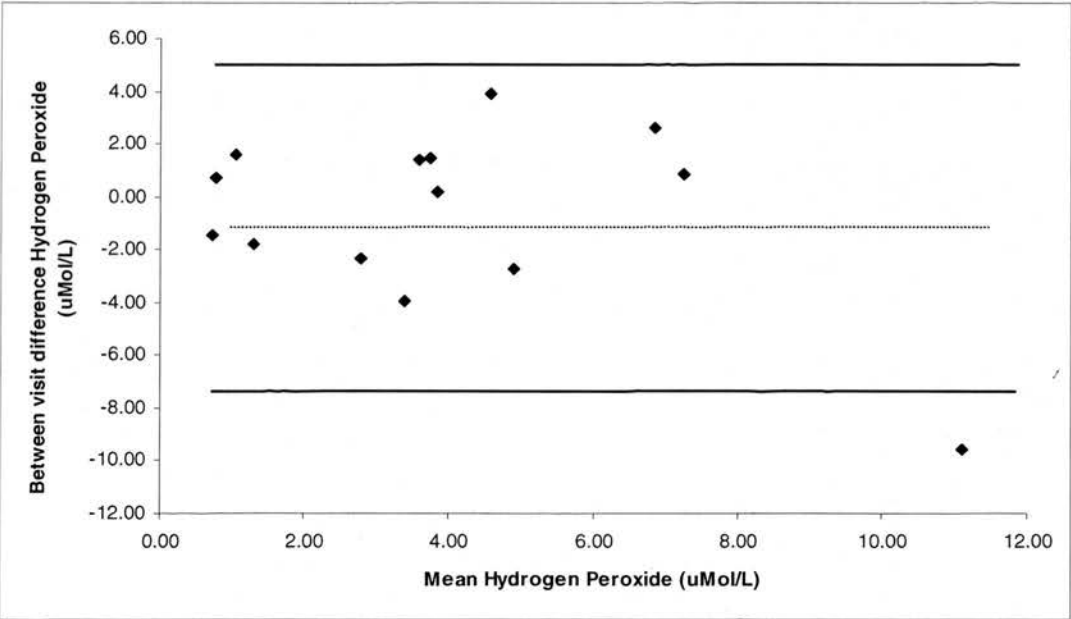
There was no significant difference in the mean levels of hydrogen peroxide measured in the three treatment groups.

The correlation coefficient for between visit measurements of hydrogen peroxide in the study population was 0.48 with a range from 0.00 to 0.77. This correlation failed to reach statistical significance with a  $p$  value of 0.057. Statistical analysis repeated after values below the limit of detection of the assay had been removed did not alter the correlation coefficient. Figure 3.13



**Figure 3.12** Dot Plot Representation of Hydrogen Peroxide levels measured during study

Plots represent Hydrogen Peroxide levels per individual during trial. Dashed vertical line represents Limit of Detection of Assay (0.78 $\mu\text{Mol/L}$ )



**Figure 3.13** Bland Altman plot Hydrogen Peroxide Levels in Exhaled Breath Condensate during study

Dot plots represent the mean of study visits for each individual subject in the x axis against the difference between study visits in the y axis. The dashed line represents the bias between visits (-0.6  $\mu\text{Mol/L}$  ) and the solid lines the limit of agreement of the test, 5.8 $\mu\text{Mol/L}$  to -7.0 $\mu\text{Mol/L}$ .

### 3.3.4 Conclusion

Levels of both biomarkers of oxidative stress were measurable in exhaled breath condensate from the majority of patients.

8-Isoprostane levels did not display Gaussian distribution with the majority of measurements being on or close to the lower limit of detection of the assay. The study was not designed to compare levels between the treatment groups and the numbers studied were small. It should be noted however that there was no significant difference in levels of 8-Isoprostane between the groups.

There was a statistically significant between-visit correlation in log 8-Isoprostane levels in this study population. Hydrogen peroxide levels were normally distributed although levels were not detectable in a minority of cases. As with 8-Isoprostane, there was no significant difference in levels between the study groups.

Hydrogen peroxide levels did demonstrate a trend toward correlation between study visits however this failed to reach statistical significance.

The results of this chapter are discussed in detail in Chapter 6.2.

## Chapter 4

### **Relationship between Exhaled Breath Condensate Biomarkers and Lung Function, Health Status and Smoking Status in Subjects with COPD and Healthy Controls**

## Chapter 4

### Relationship between Exhaled Breath Condensate Biomarkers and Lung Function, Health Status and Smoking Status in Subjects with COPD and Healthy Controls

#### 4.1 Introduction

Several biomarkers of inflammation and oxidative stress have been identified which may potentially be of use in the study of the Exhaled Breath Condensate (EBC) of patients with COPD. Published data regarding this are discussed in Chapter One of this thesis. The reliability of 8-Isoprostane and Hydrogen Peroxide in Exhaled Breath Condensate when measured by our group is discussed in Chapter Four. In this chapter, comparison is also made between levels of these biomarkers in EBC in five subject groups; COPD smokers, COPD Ex-smokers, Control Smokers, Control Ex-Smokers and Control Non-Smokers. The relationship between these biomarkers and a variety of parameters of health in subjects with COPD is also discussed. Samples obtained in this study were also used to identify novel biomarkers in EBC which may be of use in clinical studies.

##### 4.1.1 Study Population

Subjects were recruited from local secondary care clinics, GP practices and from local advertisements. Subjects were asked to give written informed consent to the trial prior to participation.

Prior to trial recruitment COPD subjects required a documented diagnosis of COPD, had to be over 40 years of age and be either a current or ex-smoker (defined as no tobacco consumption for 6 months) with a minimum of 10 pack

years tobacco exposure. A clinical diagnosis of COPD had to be supported by spirometric evidence of airflow obstruction with post-bronchodilator FEV<sub>1</sub> less than 80% predicted and FEV<sub>1</sub>/FVC ratio less than 70%. Subjects with significant improvement after bronchodilator, defined as greater than 400ml or reversal of airflow obstruction were excluded from the trial.

Study visits were only performed on clinically stable patients, defined as no exacerbation in the last month or no exacerbation requiring hospital admission in the last 2 months. An exacerbation was defined as a change in baseline dyspnoea, cough and / or sputum production sufficient to merit a change in management.

Control subjects were recruited from local GP practices and by local advertisements. Subjects were over the age of 40. Current and ex-smokers required a smoking history of 10 pack years. Ex-smokers must have abstained for at least 6 months. Non-smokers had to be either lifelong non-smokers or have a smoking history of less than 1 pack year. All subjects were to be free from any clinically significant disease which was felt may either compromise the subject's safety during the trial or interfere with the study results.

Healthy volunteers were required to have normal spirometry, as defined by both FEV<sub>1</sub> and FVC greater than 80 percent predicted with no significant reversibility and FEV<sub>1</sub>/FVC ratio greater than 70%. A number of exclusion criteria were used in this study and applied to both COPD patients and healthy controls. These included current or previous history of atopy, sinusitis, respiratory disease other than COPD, unexplained respiratory symptoms, any recent upper or lower respiratory tract infection and non-respiratory inflammatory disease (appendix 4.1) .

#### 4.1.2 Study Visits

Prior to study attendance, subjects were asked to abstain from smoking and caffeine for a minimum of 8 hours. Inhaled corticosteroids and bronchodilators were withheld on the morning of clinic attendance. Trial participation was deferred if subjects had received any antibiotics in the last 4 weeks or oral corticosteroids in the last 8 weeks.

On study attendance, clinical history was reviewed to ensure suitability for trial participation. Past medical history, drug history, occupational history and family history were recorded. Smoking history was noted including the time of the last cigarette. COPD subjects were asked to complete a St Georges Respiratory Questionnaire (appendix 5.3) and MRC Dyspnoea Score (appendix 4.2).

Symptoms of cough, sputum production and wheeze were scored as either zero (no symptoms), one (intermittent symptoms during day) or 2 (constant symptoms throughout day). The presence or absence of ankle oedema and nocturnal symptoms were recorded and annual exacerbation rate noted along with date of last exacerbation.

Height, weight, body mass index, blood pressure, oxygen saturation and pulse rate were measured. Exhaled carbon monoxide level was measured and exhaled breath condensate collection was only undertaken if there was no evidence of acute cigarette smoke exposure.

Exhaled breath condensate collected using a standardised procedure (Appendix 2.1). Specimens were frozen at  $-80^{\circ}\text{C}$  and analysed in batches using standard assay protocols (Appendix 2.6,2.7). Lung spirometry was then measured, again using a standardized procedure (Appendix 2.5).



#### **4.1.3 Statistical Analysis**

The method of statistical analysis was dependant on the distribution of the data.

Results with more than two data sets with a parametric distribution were analyzed using Tukey One Way Analysis of Variance (ANOVA). Analysis between two data sets with a parametric distribution used paired t-test. Non parametric data were analyzed using the Dunn Formula for groups and the Mann-Whitley test for two data sets.

#### **4.1.4 Ethical Approval**

Ethical approval for this study was obtained from the ethics committee of Lothian Acute Hospitals NHS Trust. Prior to trial participation, subjects were issued with written information regarding the study and given a minimum of 24 hours to digest this information. Written informed consent was given and signed prior to trial recruitment.

## 4.2 Demographics

A total of 139 subjects participated in the trial. Seventy eight of these subjects had COPD and 61 were controls. Thirty seven of the subjects with COPD were current smokers, 41 ex-smokers. Thirteen of the control subjects were current smokers, 23 ex-smokers and 25 lifelong non-smokers.

All trial participants were over forty; however the groups were not age matched. Subjects with COPD were significantly older ( $p<0.001$ ) when compared with controls. Seventy six of the subjects were male. There were statistically more males in the COPD ex-smoker group compared with the control smokers and control non-smoker groups.

COPD ex-smokers were older than current smokers ( $p<0.01$ ). COPD subjects had a significantly higher smoking pack year history compared with healthy smokers,  $p<0.001$ . There was no difference in the pack years between COPD ex and current smokers. Equally there was no difference in pack years between control ex or current smokers.

There was no statistically significant difference in body mass index between the groups. The COPD groups had significantly higher levels of cardiovascular disease compared with the control group. Fourteen of the 78 subjects with COPD also had ischaemic heart disease compared with 3 of the healthy controls,  $p<0.05$ . Twenty four COPD subjects had hypertension compared with six of the 61 healthy controls,  $p<0.05$ . Table 4.1

Subjects with COPD had significantly lower  $FEV_1$  ( $p<0.001$ ) compared with controls, with no differences between the two COPD groups or between the three

control groups. Control ex-smokers ( $p<0.01$ ) and non-smokers ( $p<0.05$ ) had higher mean FVC compared with COPD subjects. There was no difference between COPD subjects and current smokers and no difference between the two COPD groups.

COPD subjects had a lower  $FEV_1/FVC$  ratio compared with controls,  $p<0.001$ . There was no difference between the two COPD groups or between the three control groups. COPD current smokers demonstrated more reversibility to bronchodilator,  $155\text{ml} \pm 167$  when compared with healthy non-smokers,  $48\text{ml} \pm 93$ ,  $p<0.05$ , there was no difference between the other groups in terms of reversibility.

Table 4.4

COPD ex-smokers had statistically lower total St Georges Respiratory Questionnaire score compared with COPD current smokers,  $42.7 \text{ points} \pm 20.0$  versus  $58.2 \text{ points} \pm 15.9$ ,  $p<0.001$ . All components of the SGRQ score were lower in the ex-smoking group.

Current smokers with COPD were more likely to report symptoms of chronic bronchitis (80% versus 30%,  $p<0.001$ ) and had higher mean cough scores ( $1.5 \pm 0.6$  versus  $1.1 \pm 0.8$ ,  $p<0.05$ ) compared with COPD ex-smokers. There were no differences in symptoms of breathlessness, wheeze or exacerbation frequency. There was no difference in the use of any form of bronchodilator between the two groups. COPD ex-smokers were more likely to be on inhaled corticosteroids, 32 out of 41 compared with 19 of 37 COPD current smokers,  $p<0.05$ .

Group	COPD Smoker	COPD Ex	Healthy Smoker	Healthy Ex	Healthy Non
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Number	37	41	13	23	25
Age mean $\pm$ sd	64.6 $\pm$ 8.4	72.1 $\pm$ 6	55.6 $\pm$ 9.3	59.7 $\pm$ 10.2	54.0 $\pm$ 9.0
Sex (m/f)	21/15	32/9	4/8	11/11	8/17
BMI mean $\pm$ sd	24.2 $\pm$ 4.2	26.8 $\pm$ 5.8	27.9 $\pm$ 3.4	27.1 $\pm$ 4.2	27.0 $\pm$ 4.6
FFM Mean $\pm$ sd	46.7 $\pm$ 10.5	55.2 $\pm$ 10.4	49.1 $\pm$ 13.7	51.7 $\pm$ 10.6	50.6 $\pm$ 9.0
Pack Years mean $\pm$ sd	49.5 $\pm$ 23.7	45.8 $\pm$ 18.8	26.9 $\pm$ 12.7	23.9 $\pm$ 10.8	0.3 $\pm$ 1.1
IHD	7	7	1	2	0
HBP	10	14	1	4	1
High Cholesterol	2	1	1	2	2
Diabetes	1	2	0	0	1

**Table 4.1 Patient Demographics**

BMI= Body mass index, FFM= Fat Free Mass, IHD= Ischaemic Heart Disease, HBP= High Blood Pressure

Group	COPD Smoker	COPD Ex	Healthy Smoker	Healthy Ex	Healthy Non
FEV <sub>1</sub> Post BD (L) Mean ± sd	1.3 ± 0.6	1.4 ± 0.6	2.8 ± 0.6	3.0 ± 0.6	3.1 ± 0.7
FVC Post BD (L) Mean ± sd	3.0 ± 3.1	2.9 ± 1.0	3.6 ± 0.9	4.0 ± 0.8	3.8 ± 1.0
FEV <sub>1</sub> /FVC Ratio Mean ± sd	51 ± 15	49 ± 13	78 ± 9	77 ± 8	81 ± 7
FEV <sub>1</sub> Post BD (%Pred) Mean ± sd	52.3 ± 22	53.0 ± 21	100 ± 15	104 ± 14	108 ± 12
%change post BD Mean ± sd	15 ± 15	13 ± 12	3 ± 7	3 ± 5	2 ± 3
Vol.Change postBD Mean ± sd	155±167	137±120	68±153	95±158	48±93

**Table 4.2 Spirometry Results per Study Group**

FEV<sub>1</sub>=Forced Expiratory Volume in 1 sec, FVC=Forced Vital Capacity,  
BD=bronchodilator

Group	COPD Smoker	COPD Ex-Smoker
Number	37	41
MRC Chronic Bronchitis Mean $\pm$ Sd	0.8 $\pm$ 0.4	0.3 $\pm$ 0.5
MRC Dyspnoea Mean $\pm$ Sd	3.0 $\pm$ 0.9	2.8 $\pm$ 1.0
Cough (0-3) Mean $\pm$ Sd	1.5 $\pm$ 0.6	1.1 $\pm$ 0.8
Sputum (0-2) Mean $\pm$ Sd	1.6 $\pm$ 0.6	1.7 $\pm$ 0.7
Wheeze (0-2) Mean $\pm$ Sd	1.2 $\pm$ 0.8	0.9 $\pm$ 0.7
Exac Freq Mean $\pm$ Sd	1.8 $\pm$ 1.3	2.1 $\pm$ 2.3

Table 4.3 Health Status Results per study group

Group	COPD Smoker	COPD Ex Smoker
Number	37	41
SGRQ Total Mean $\pm$ Sd	58.2 $\pm$ 15.9	42.7 $\pm$ 20.0
SGRQ Symptoms Mean $\pm$ Sd	73.5 $\pm$ 18.3	55.0 $\pm$ 23.1
SGRQ Activity Mean $\pm$ Sd	72.5 $\pm$ 19.5	59.5 $\pm$ 23.4
SGRQ Impact Mean $\pm$ Sd	45.1 $\pm$ 18.6	28.9 $\pm$ 20.7

Table 4.4 St Georges Respiratory Questionnaire in Current and Ex-Smokers with COPD

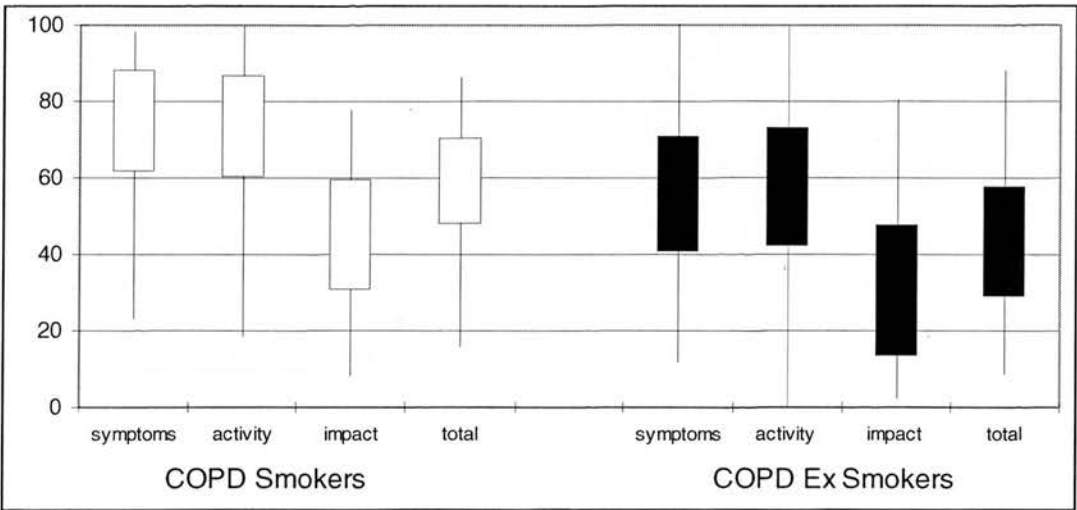


Figure 4.1 St Georges Health Questionnaire in COPD Smokers and Ex-smokers.

White Histograms represent COPD current smokers, black histograms COPD Ex-smokers. Higher mean scores were recorded in current smokers for symptoms ( $p<0.001$ ), activity ( $p<0.001$ ) and impact ( $p<0.001$ ) component scores. Total mean score was significantly higher in the current smokers ( $p<0.001$ ).

Group	COPD Smoker	COPD Ex Smoker
Number	37	41
SABA	30	32
LABA	13	21
SAAC	11	10
LAAC	11	10
ICS	19	32
Theophylline	2	0

**Table 4.5 COPD Treatment in Current and Ex Smoking Groups**

SABA= short acting  $\beta$  agonist, LABA= long acting  $\beta$  agonist, SAAC= short acting anti-cholinergic, LAAC= long acting anti-cholinergic, ICS= Inhaled Corticosteroid



### 4.3 Measurement of Biomarkers in Exhaled Breath Condensate Relationship with COPD and Smoking

No significant difference was demonstrated in mean levels of 8-Isoprostane measured in exhaled breath condensate between subjects with COPD and controls. There were also no significant differences between groups dependant on current smoking status. Figure 4.2

Mean hydrogen peroxide levels in EBC were higher in ex-smokers with COPD ( $3.5\mu\text{mol/L} \pm 0.9$ ) compared with current smokers with COPD ( $2.0\mu\text{mol/L} \pm 0.5$ ),  $p=0.007$ . Mean hydrogen peroxide levels in current smokers with COPD were similar to the three healthy control groups. Ex-smokers with COPD had higher levels than healthy non-smokers ( $3.5\mu\text{mol/L} \pm 0.9$  versus  $1.1\mu\text{mol/L} \pm 0.2$ ,  $p=0.0003$ ) but not healthy smokers or ex-smokers. Figure 4.3

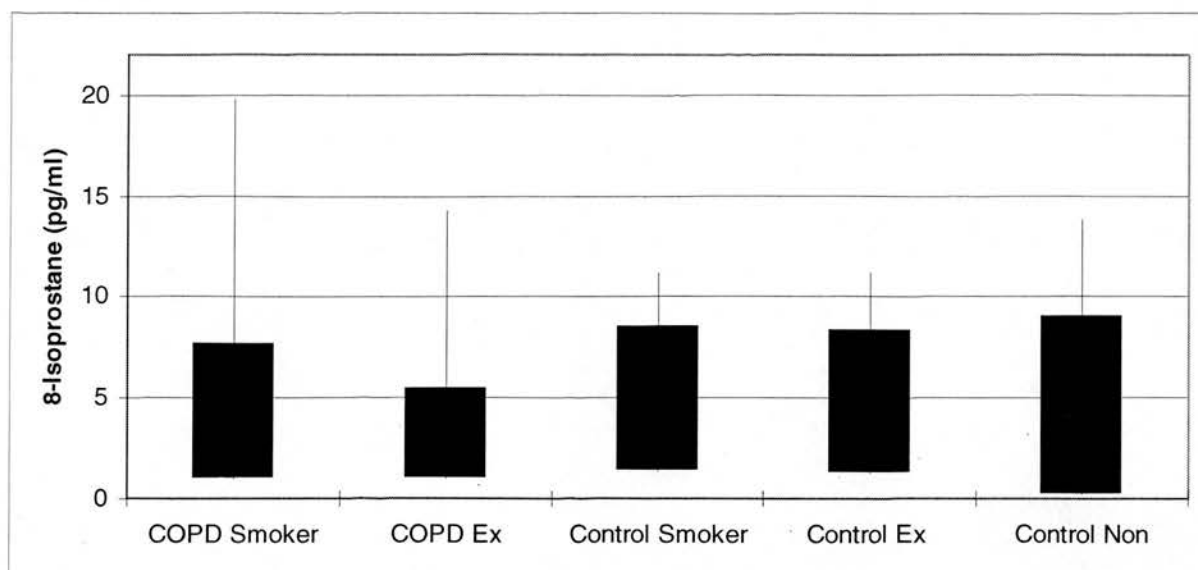
Ex-smokers with no evidence of respiratory disease had elevated hydrogen peroxide levels compared with control non-smokers ( $2.0\mu\text{mol/L} \pm 0.4$  versus  $1.1\mu\text{mol/L} \pm 0.2$ ,  $p=0.048$ ). Current smokers without respiratory disease had lower levels than ex-smokers. However this was not statistically significant ( $p>0.05$ ).

Overall, independent of current smoking habit, subjects with COPD had higher mean hydrogen peroxide levels ( $2.8\mu\text{mol/L} \pm 0.5$ ) compared with subjects without COPD ( $1.5\mu\text{mol/L} \pm 0.2$ ),  $p=0.04$ . Figure 4.4

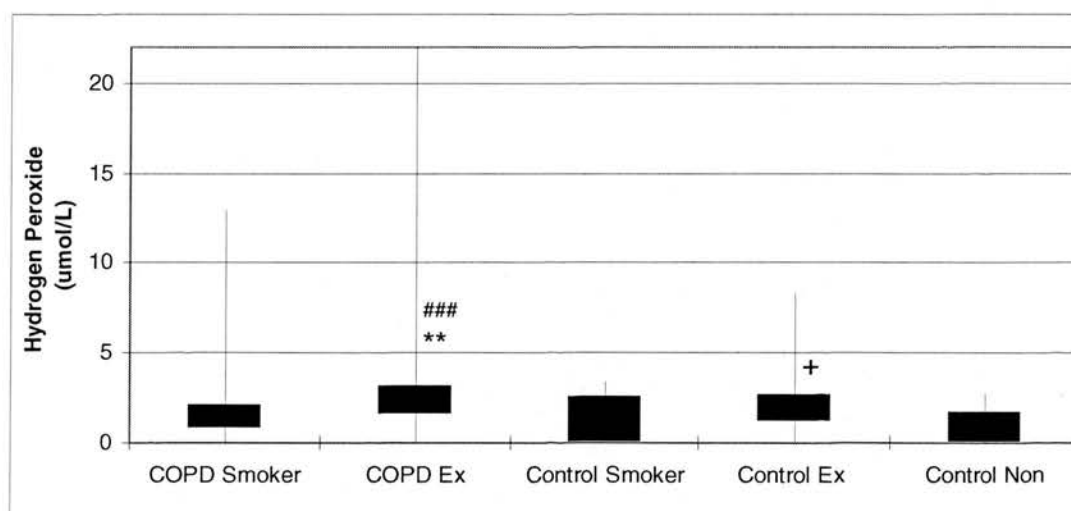
When current smoking intake was analysed independent of respiratory disease, ex-smokers had higher levels of hydrogen peroxide ( $3.0\mu\text{mol/L} \pm 0.6$ ) compared with current smokers ( $1.9\mu\text{mol/L} \pm 0.4$ ),  $p=0.0134$  and lifelong non-smokers ( $1.1\mu\text{mol/L} \pm 0.2$ ),  $p=0.0007$ . Figure 4.4

A variety of other biomarkers were identified as being potentially measurable in COPD. These included Leukotriene B<sub>4</sub>, Prostaglandin E<sub>2</sub>, pH, Myeloperoxidase, Tumour Necrosis Factor  $\alpha$  and Interleukins 6,8 and 10. Of these biomarkers only Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and pH could be readily detected in EBC on a regular basis.

Prostaglandin E<sub>2</sub> levels did not significantly vary between the two COPD groups. Levels in the control smokers and ex-smokers groups were similar to those in the COPD groups. Control non-smokers had significantly lower mean levels of PGE<sub>2</sub> (9.6pg/ml  $\pm$  1.0, p=0.03) compared with current smokers (12.2pg/ml  $\pm$  0.7) and healthy ex-smokers (12.4pg/ml  $\pm$  0.8, p=0.03). Subjects with COPD, independent of current smoking habit, had similar PGE<sub>2</sub> levels (11.7pg/ml  $\pm$  0.5) when compared with healthy current or ex-smokers (12.2pg/ml  $\pm$  0.8). The mean levels of PGE<sub>2</sub> in healthy non-smokers was significantly lower than both these groups (both p values =0.04). Figures 4.5, 4.6. No significant variation was seen in the pH of EBC in the five study groups. Figure 4.7

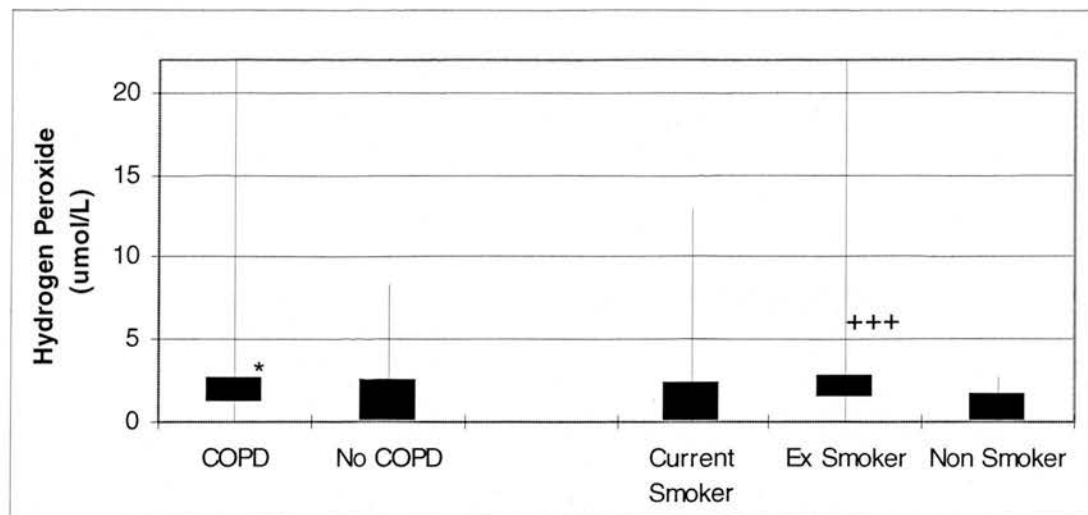


**Figure 4.2 Levels of 8-Isoprostane measured in Exhaled Breath Condensate per Study Group.** Histograms represent Box and whisker plots of quartiles of 8-Isoprostane levels (pg/ml). No significant difference is noted between the five study groups.



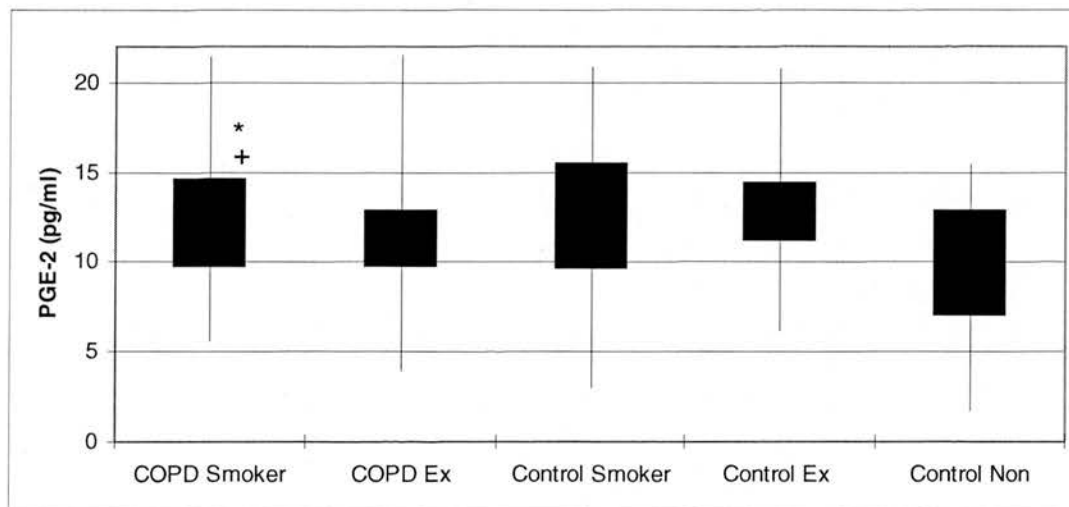
**Figure 4.3 Levels of Hydrogen Peroxide measured in Exhaled Breath Condensate per Study Group**

Histograms represent Box and whisker plots of quartiles of hydrogen peroxide levels ( $\mu\text{mol/L}$ ) for the five study groups. Significantly higher mean levels of hydrogen peroxide in COPD Ex-smokers compared with COPD current smokers,  $p=0.007(**)$ , and control non-smokers,  $p=0.0003(###)$ . Control ex-smokers had higher levels of hydrogen peroxide compared with control non-smokers,  $p=0.048(+)$ .



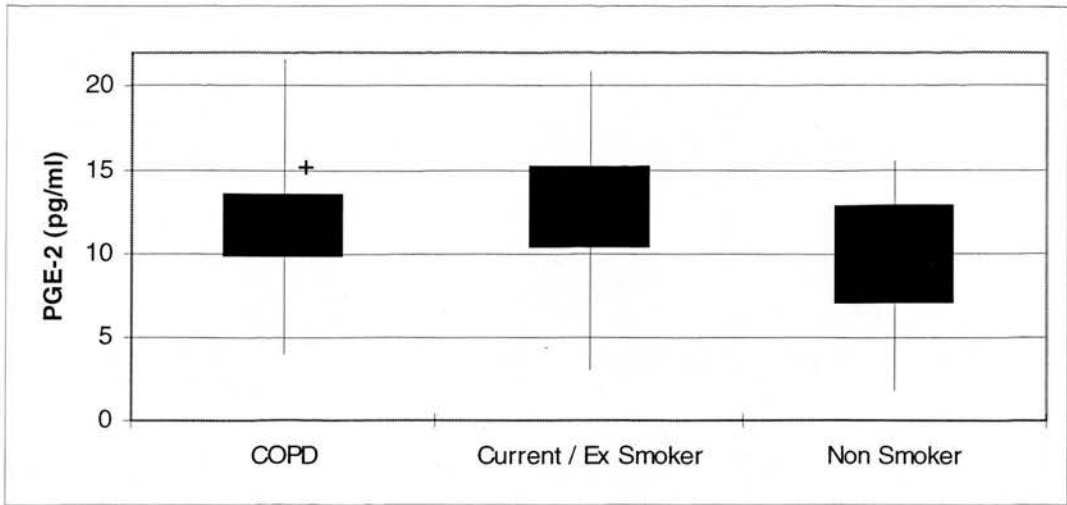
**Figure 4.4** Hydrogen Peroxide levels in Exhaled Breath Condensate in subjects with COPD compared with controls and in Current, Ex Smoking and Non Smoking Groups Independent of Respiratory Disease.

Histograms represent Box and whisker plots of quartiles of hydrogen peroxide levels ( $\mu\text{mol/L}$ ) for the subjects with and without COPD independent of smoking status and for smokers, ex-smokers and lifelong non-smokers independent of respiratory disease. Significantly higher levels of hydrogen peroxide were measured in subjects with COPD compared with those without,  $p=0.04$  (\*). Ex-smokers had higher hydrogen peroxide levels than non-smokers,  $p=0.0007$  (+++) with a trend towards higher levels compared with current smokers,  $p=0.013$ .



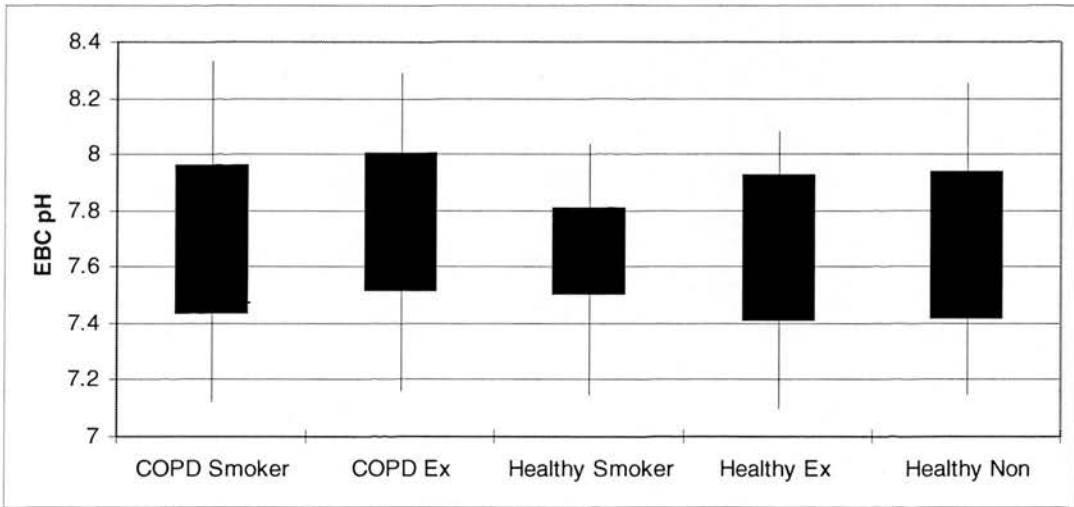
**Figure 4.5** PGE2 levels in Exhaled Breath Condensate per study group

Histograms represent Box and whisker plots of the four quartiles of PGE2 levels (pg/ml) in EBC. Significantly higher levels were seen in COPD smokers,  $p=0.03$ , (\*) and control Ex-smokers,  $p=0.03$  (+) compared with non smoking controls.



**Figure 4.6** PGE2 levels in Exhaled Breath Condensate per study group

Histograms represent Box and whisker plots of the four quartiles of PGE2 levels (pg/ml) in EBC. Significantly higher levels were seen in COPD patients,  $p=0.04$  (\*), and Control Smokers or Ex-smokers,  $p=0.04(+)$ , compared with non smoking controls.



**Figure 4.7** pH of Exhaled Breath Condensate per study group

Histograms represent Box and whisker plots of the four quartiles of EBC pH. No significant variation is demonstrated between the five study groups.

## **4.4 Relationship between biomarkers and Co-Morbidity other than COPD**

Section 4.3 demonstrated that levels of many of the biomarkers were similar in subjects with COPD compared with the control population. One possible explanation for this is that the control population has a significant degree of non-respiratory co-morbidity which may contribute to elevated levels of exhaled breath condensate biomarkers.

To further investigate this possibility, the control population was divided into those subjects with or without significant non respiratory disease. Twenty of the 61 subjects in the control group had no significant co-morbidity, compared with 41 with non-respiratory co-morbidity. Co-morbidities of the trial participants are listed in appendix 4.3. There was no significant difference in smoking status between control subjects with and without co-morbidity. Table 4.6

Analysis of the 4 biomarkers was performed comparing control subjects with no co-morbidity to subjects with any co-morbidity including COPD. Further analysis was performed comparing subjects with a non respiratory co-morbidity to subjects with COPD.

### **4.4.1 pH and Co-Morbidity**

No significant differences in mean levels of pH in EBC were noted between subjects with and without significant co-morbidity. Figure 4.8

### **4.4.2 PGE2 and Co-Morbidity**

Prostaglandin E2 levels in EBC of subjects with no co-morbidity ( $9.8 \text{ pg/ml} \pm 0.9$ ) demonstrated a trend towards lower mean levels compared with subjects with any co-morbidity including COPD ( $11.6 \pm 0.4$ ,  $p=0.08$ ) and non COPD co-morbidity ( $12.1 \text{ pg/ml} \pm 0.9$ ,  $p=0.08$ ). Mean levels in the COPD group as a whole also

demonstrated a trend towards higher levels ( $11.6\text{pg/ml} \pm 0.5$ ,  $p=0.07$ ) compared with controls having no co-morbidity. The difference when compared with the control group without co-morbidity was statistically significant in current smokers with COPD ( $12.2\text{pg/ml} \pm 0.7$ ,  $p=0.04$ ) Figure 4.10

#### **4.4.3 Hydrogen Peroxide and Co-Morbidity**

There was no significant difference between hydrogen peroxide levels in EBC of controls with no co-morbidity to all of the other subject groups. Figure 4.9

#### **4.4.4 8-Isoprostane and Co-Morbidity**

8-Isoprostane levels were significantly lower in control subjects with no co-morbidity ( $3.4\text{pg/ml} \pm 0.5$ ) compared with subjects with any co-morbidity ( $5.9\text{pg/ml} \pm 0.4$ ,  $p=0.02$ ) and subjects with non COPD co-morbidity ( $7.0\text{pg/ml} \pm 0.7$ ,  $p=0.001$ ). There was no significant difference between control subjects with no co-morbidity and subjects with COPD. There was however a trend towards higher levels in current smokers with COPD ( $5.6\text{pg/ml} \pm 0.8$ ) compared with the group with no co-morbidity,  $p=0.09$ . Figure 4.11

	Control		COPD
	No Comorbidity	Co morbidity	
Current Smoker	4	9	37
Ex Smoker	7	16	41
Non Smoker	9	16	
Total	20	41	78

Table 4.6      Smoking status of subjects related to co-morbidities

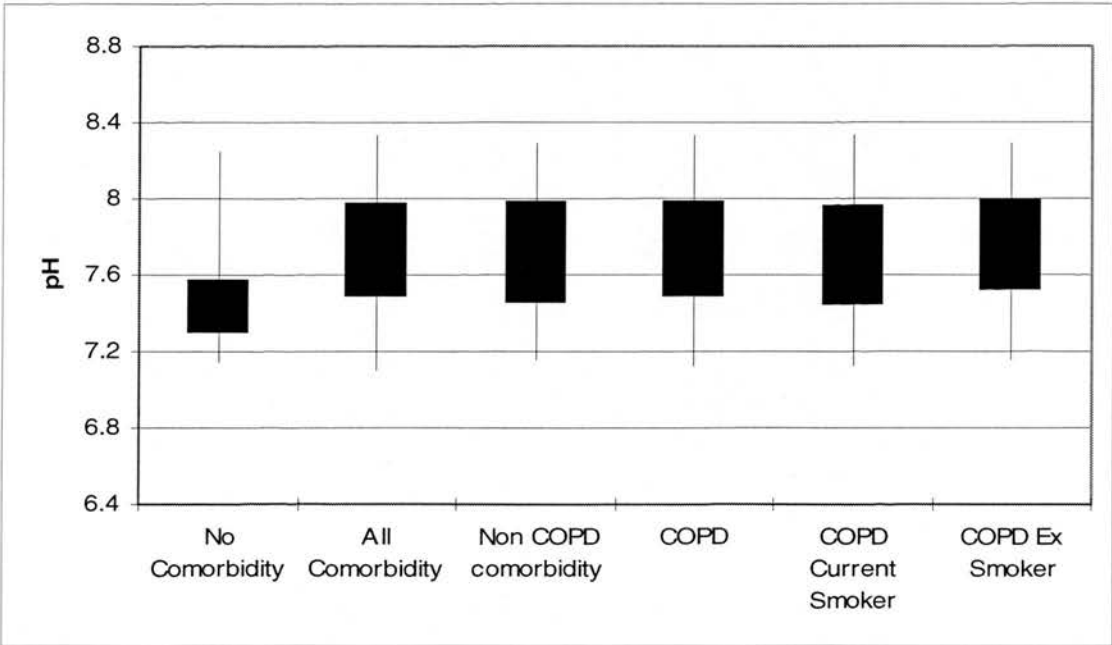
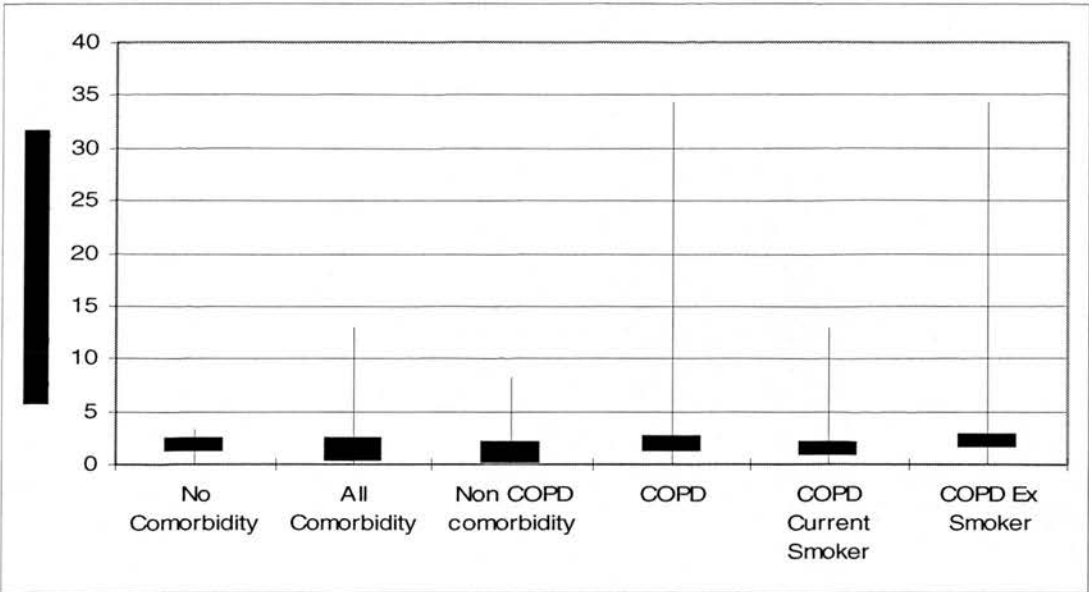


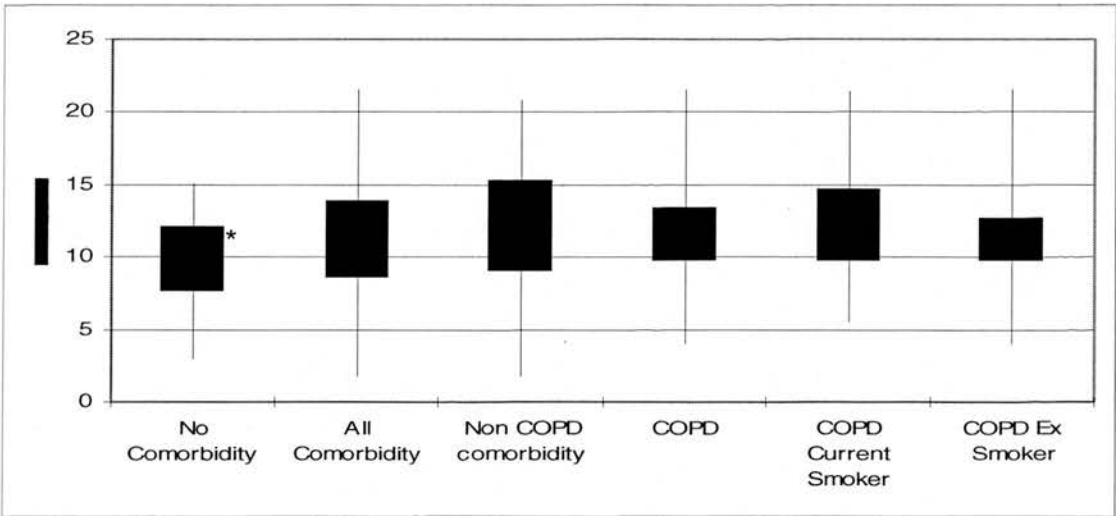
Figure 4.8      pH levels in EBC compared withSubject Co-Morbidity      Histograms represent Box and whisker plots of quartiles of pH levels in trial subjects. No significant difference was noted between any of the subject groups.





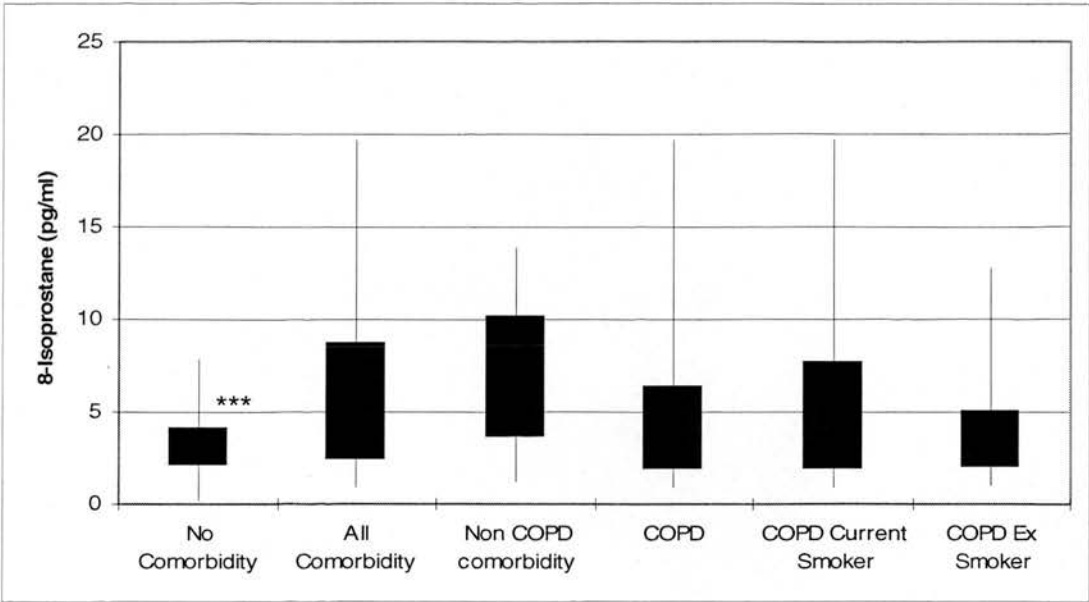
**Figure 4.9 Hydrogen Peroxide Levels in EBC per Co-Morbidity**

Histograms represent Box and whisker plots of the quartiles of EBC hydrogen peroxide levels per subject co-morbidity. No significant difference was seen between subjects with no co-morbidity and any of the other groups.



**Figure 4.10 PGE2 Levels in EBC compared with Subject Co-Morbidity**

Histograms represent Box and whisker plots of the quartile of PGE2 levels in EBC for the trial subjects dependant on co-morbidity. Subjects with no co-morbidity had significantly lower levels of PGE2 ( $9.8\text{pg/ml} \pm 0.9$ ) compared with Current Smokers with COPD ( $12.2\text{pg/ml} \pm 0.7$ )  $p=0.04$  (\*).



**Figure 4.11 8-Isoprostane levels in EBC per Subject Co-morbidity**

Histograms represent Box and whisker plots of the quartiles of 8-isoprostane levels in EBC per co-morbidity. Levels of 8-Isoprostane in EBC were significantly higher in subjects with all causes of co-morbidity ( $5.9\text{pg/ml} \pm 0.4$ ,  $p=0.02$ ) and non COPD co-morbidity ( $7.0\text{pg.ml} \pm 0.7$ ,  $p=0.001$ , \*\*\*). 8-Isoprostane levels in COPD current smokers demonstrated a trend towards higher levels ( $5.6\text{pg/ml} \pm 0.8$ ) compared with healthy controls,  $p=0.09$ .

#### **4.5 Relationship between EBC, Spirometry and Health Status in Current Smokers with COPD.**

Exhaled breath condensate was collected in 37 current smokers with COPD. The levels of Hydrogen Peroxide, 8-Isoprostane, Prostaglandin E2 and pH measured in these samples were compared with a variety of markers of health status. These included current respiratory symptoms, St Georges Health Questionnaire, COPD therapy, pack year history and lung function. Nutritional status, age and sex were also compared with EBC biomarker measurement. Listed below are the markers of health status which were compared with exhaled breath condensate biomarkers.

In summary, there were no relationships demonstrated between symptom score, past medical history, spirometry and St Georges Respiratory Questionnaire and any EBC biomarkers. Inhaled corticosteroid use was the only treatment which demonstrated a relationship to EBC, with lower levels of 8-Isoprostane in smokers with COPD compared with smokers with COPD not taking ICS. BMI was the only demographic parameter which appeared to affect biomarker measurement with higher levels in those subjects with BMI higher or lower than normal.

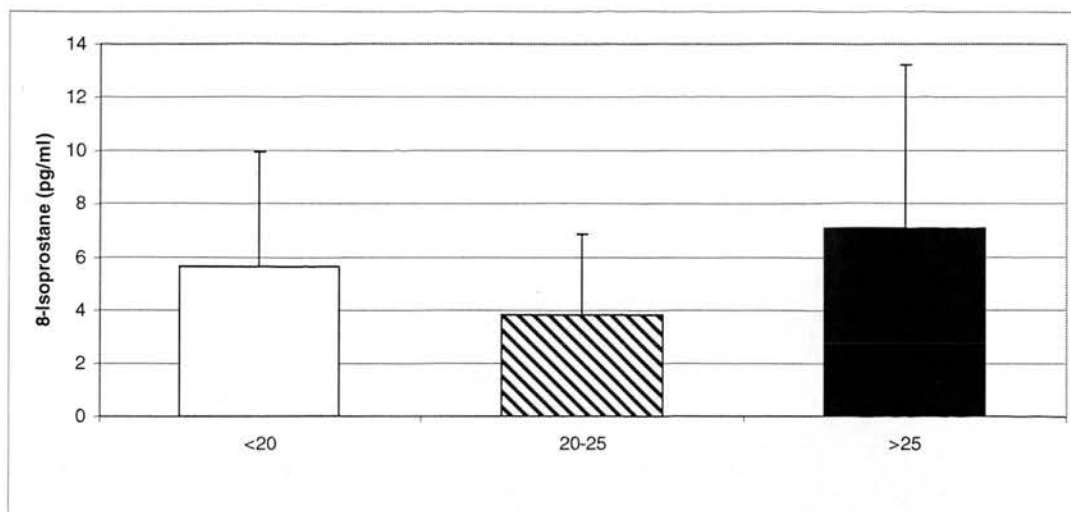
##### **4.5.1 Relationship with Biomarkers and BMI in Smokers with COPD**

Subjects with a normal Body Mass Index (BMI), between 20 and 25, demonstrated a trend towards lower mean levels of 8-Isoprostane compared with subjects with a BMI greater than 25,  $3.8\text{pg/ml} \pm 3.0$  versus  $7.1\text{pg/ml} \pm 6.1$ ,  $p=0.10$ . There was no difference between subjects with a low BMI and those with a normal BMI. Figure 4.12

Hydrogen peroxide levels also demonstrated a trend toward lower levels in those subjects with a normal BMI. With this biomarker those subjects with a BMI less than 20 had higher mean levels,  $3.7\mu\text{mol/L} \pm 4.5$  compared with those with a

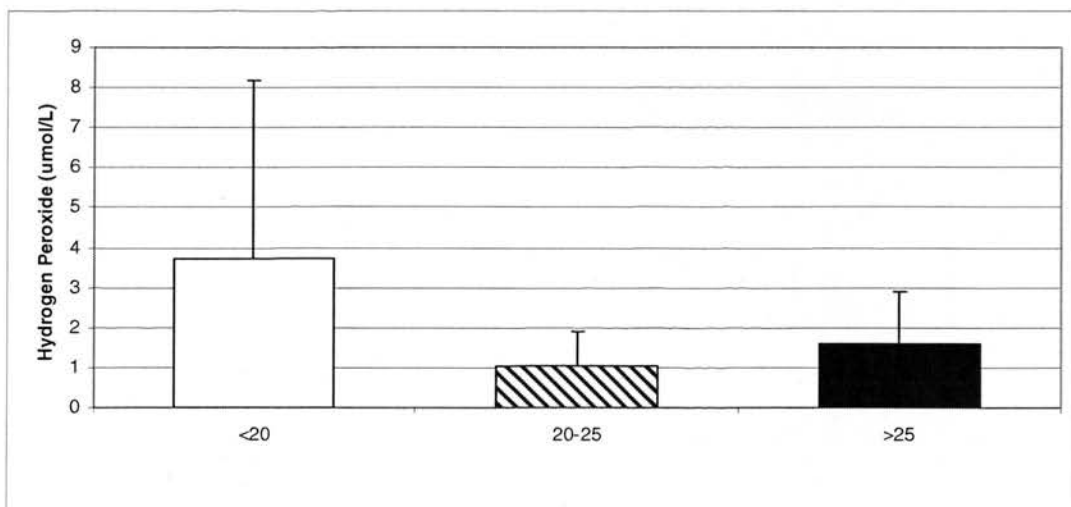
normal BMI,  $1.0 \mu\text{mol/L} \pm 0.9$ , although this was not statistically significant,  $p=0.10$ .

Figure 4.13. No relationship was demonstrated between levels of PGE2 or pH in Exhaled Breath Condensate and BMI.



**Figure 4.12 EBC 8-Isoprostane Levels in COPD Smokers Related to BMI**

White Histograms represent BMI<20, Hatched BMI 20-25 and Black BMI >25. Error Bars represent standard deviation. No statistically significant differences were demonstrated although a trend towards higher mean 8-Isoprostane levels with BMI > 25 compared with BMI 20-25 was noted,  $p=0.1$ .



**Figure 4.13 EBC Hydrogen Peroxide Levels in COPD Smokers Related to BMI**

White Histograms represent BMI<20, Hatched BMI 20-25 and Black BMI >25. Error bars represent standard deviation. No statistically significant difference was demonstrated although a trend towards higher mean hydrogen peroxide levels with BMI <20 compared with BMI 20-25,  $p=0.1$ .

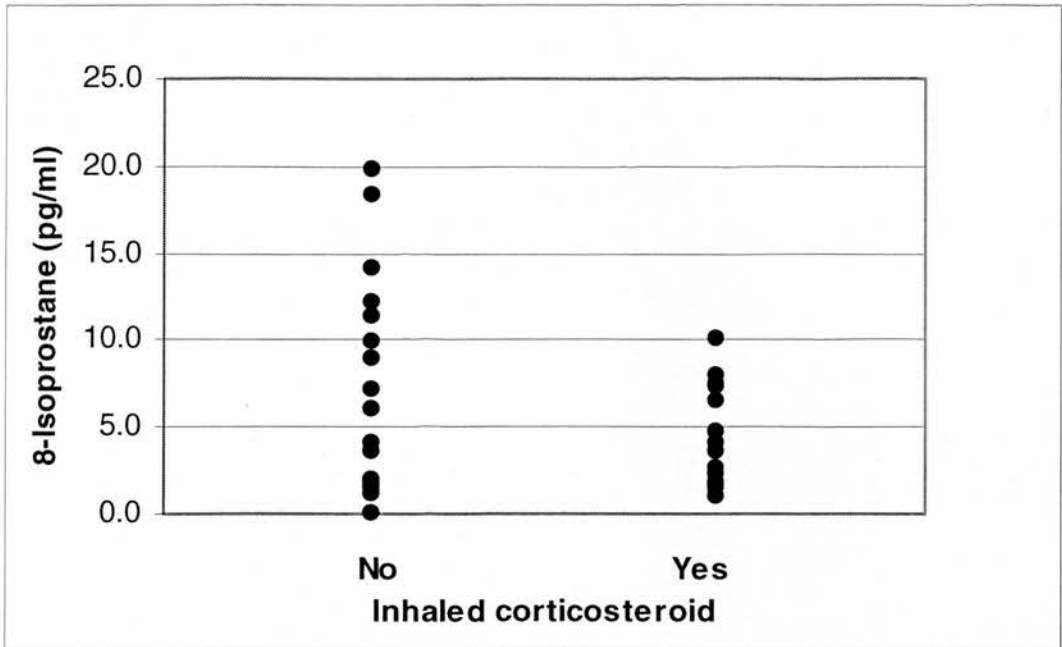
#### 4.5.2 Relationship between Biomarkers and Inhaled Corticosteroids in Smokers with COPD

Current smokers with COPD who were not taking inhaled corticosteroids on a regular basis had higher mean levels of 8-Isoprostane ( $7.4 \text{ pg/ml} \pm 6.0$ ) compared with patients using ICS ( $3.9 \text{ pg/ml} \pm 3.0$ ). This difference was statistically significant,  $p < 0.05$ . Figure 4.14

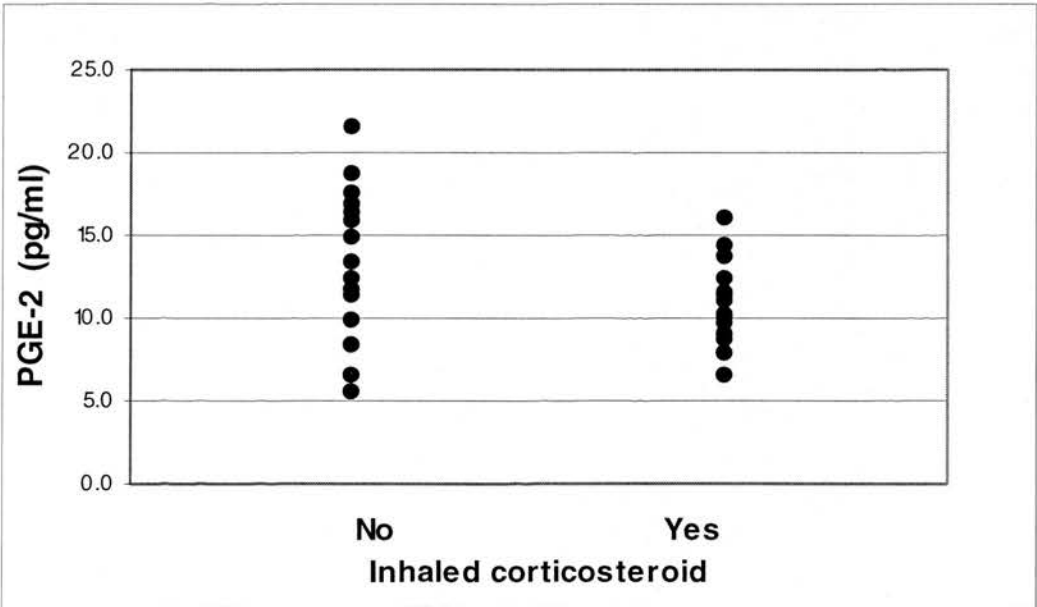
A similar relationship was demonstrated in levels of PGE2 with mean levels higher in steroid naïve subjects,  $13.5 \text{ pg/ml} \pm 4.5$  compared with those subjects using ICS,  $10.8 \text{ pg/ml} \pm 2.6$ . This relationship was close to statistical significance,  $p = 0.056$ .

Figure 4.15

No relationships were demonstrated between levels of hydrogen peroxide and pH in EBC and inhaled corticosteroid use. There was no relationship between other therapies in COPD and any of the four biomarkers.



**Figure 4.14** Inhaled Corticosteroid Use and EBC 8-Isoprostane levels in COPD Smokers Dot plots represent each individual 8-Isoprostane level in current smokers with COPD. Statistically higher mean 8-Isoprostane level in COPD Smokers not on ICS ( $7.4 \pm 6.0 \text{ pg/ml}$ ) compared with subjects on ICS ( $3.9 \pm 3.0 \text{ pg/ml}$ ).  $p < 0.05$ .



**Figure 4.15** Inhaled Corticosteroid Use and EBC PGE2 levels in COPD Smokers Dot plots represent each individual 8-Isoprostane level in current smokers with COPD. Trend towards higher PGE2 levels in COPD Smokers not on inhaled corticosteroids ( $13.5 \pm 4.5 \text{ pg/ml}$ ) compared with subjects taking ICS ( $10.8 \pm 2.6 \text{ pg/ml}$ ),  $p = 0.056$

#### 4.6 Relationship between EBC Biomarkers and Symptoms, Health Status, Therapy, Demographics, Past Medical History and Spirometry in Ex-smokers with COPD

In contrast to current smokers with COPD, when the various parameters of health in ex-smokers with COPD were compared with biomarkers of oxidative stress and inflammation in exhaled breath condensate, several relationships were noted.

Exhaled Breath Condensate biomarkers did not relate to patient demographics, past medical history or treatment. Several relationships were noted however between health status, symptom score and spirometry measurement with 8-Isoprostane levels in EBC. Hydrogen peroxide related to some measurements of lung function. There was no relationship between PGE2 and pH measurement and any of the parameters of health used.



#### 4.6.1 Relationship between 8-Isoprostane in EBC and St Georges Respiratory Questionnaire in Ex-smokers with COPD

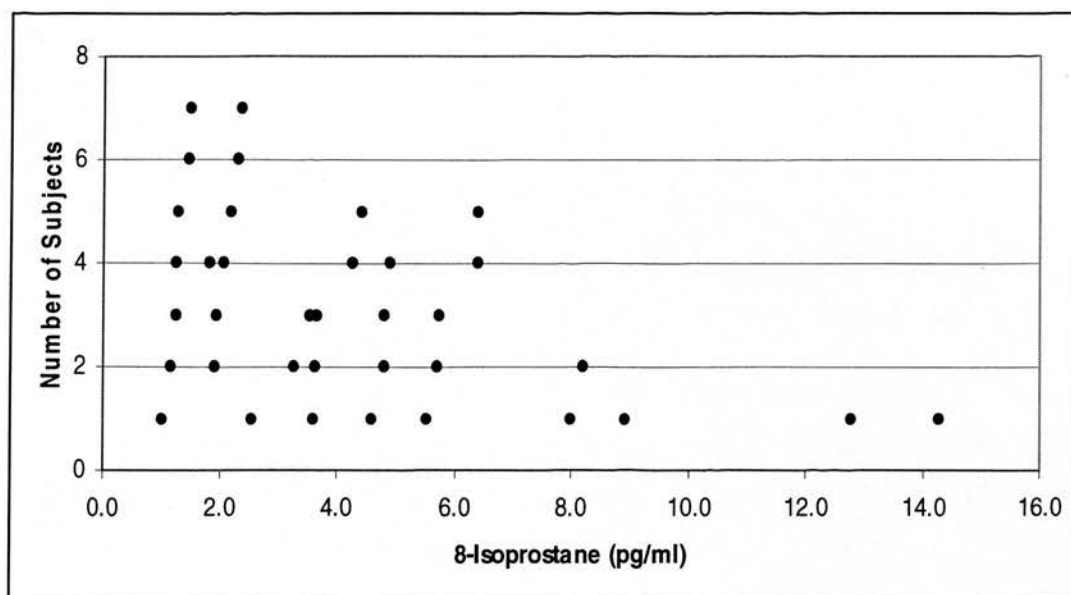
8-isoprostane levels in EBC did not display Gaussian distribution, with levels being skewed to the left, figure 4.16. Comparison of levels of oxidative stress in EBC and health status was therefore carried out by dividing the subjects by 8-Isoprostane quartile and then comparing the health status in the four groups.

Symptom score in subjects with the lowest quartile of 8-Isoprostane levels had significantly lower mean scores when compared with those subjects from the highest quartile. 42.1 points  $\pm$  5.3 versus 69.7 points  $\pm$  5.3,  $p=0.007$ , Figure 4.17.

Mean SGRQ activity scores were also higher in the highest quartile of 8-Isoprostane compared with the lowest quartile, 74.1 points  $\pm$  5.3 versus 50.0 points  $\pm$  6.4,  $p=0.02$ . Figure 4.18

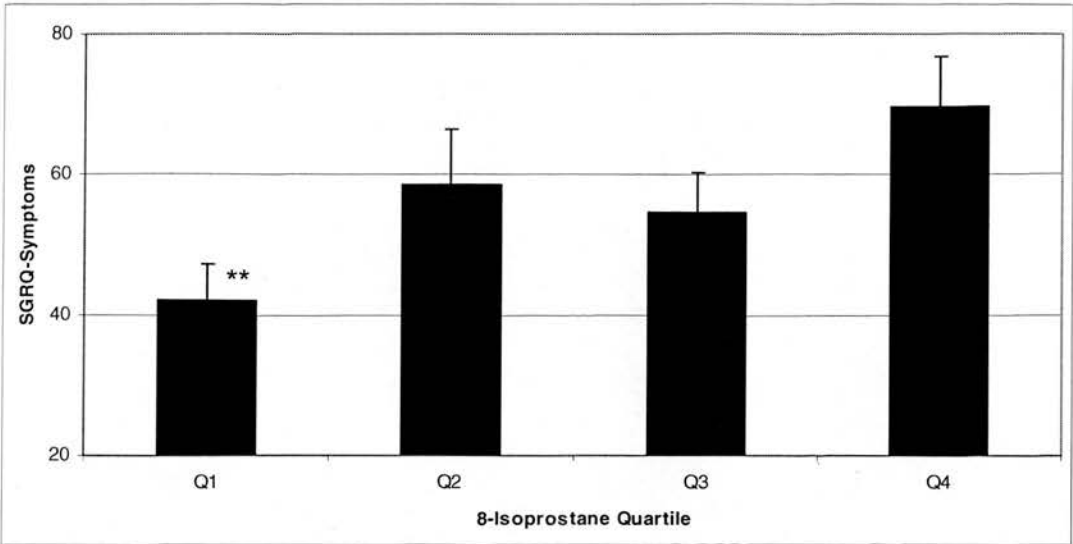
A similar relationship was also shown between Impact scores and 8-Isoprostane levels, 21.7 points  $\pm$  4.9 in the lowest 8-Isoprostane quartile compared with 43.0 points  $\pm$  6.4,  $p=0.02$ . Figure 4.19

In keeping with the three components of the SGRQ health questionnaire relating to levels of 8-Isoprostane in EBC, the mean total SGRQ score was significantly higher in those subjects with the highest 8-Isoprostane levels (57.0 points  $\pm$  6.0) compared with those with the lowest levels (33.9 points  $\pm$  4.5),  $p=0.007$ , Figure 4.20.



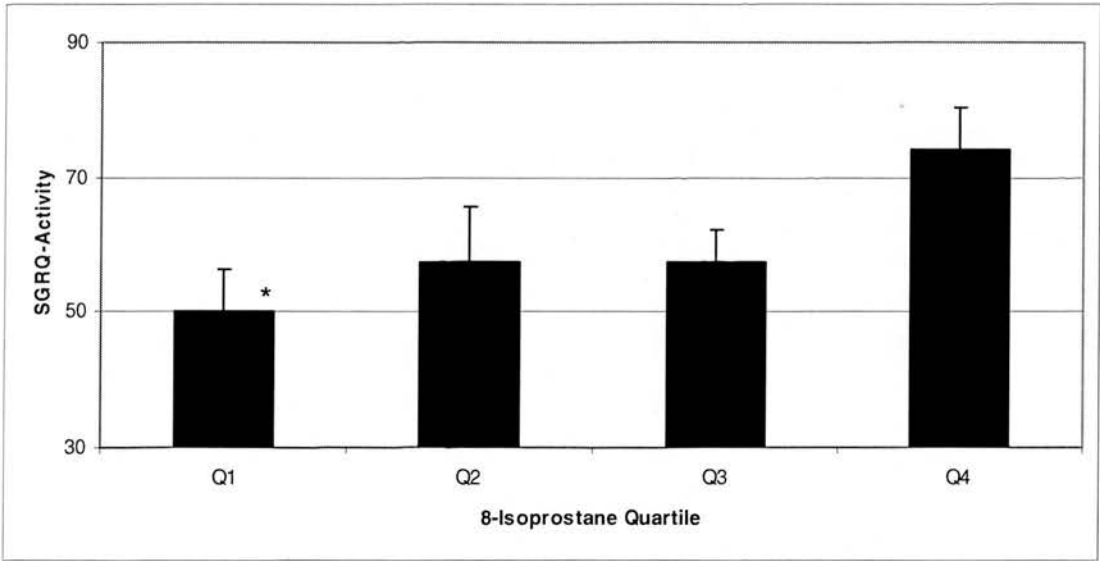
**Figure 4.16** Distribution of 8-Isoprostane measurement in EBC

Level of 8-Isoprostane measured in EBC in ex-smokers with COPD during trial. Each dot plot represents individual 8-Isoprostane measurement



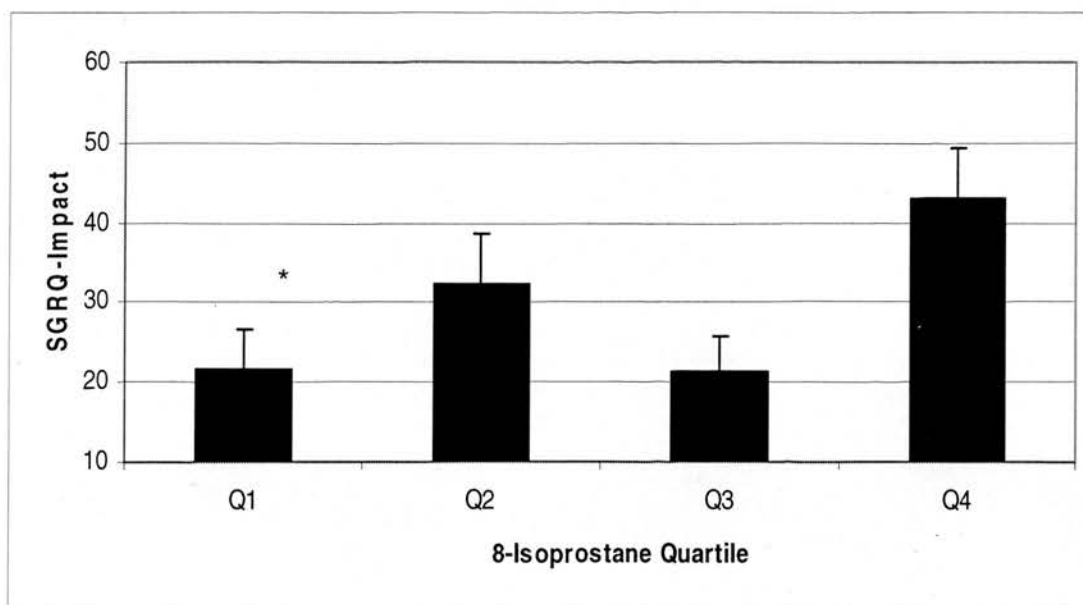
**Figure 4.17 SGRQ Symptoms Score per EBC 8-Isoprostane Quartile in COPD Ex-smokers**

Histograms represent the mean SGRQ symptom score per Quartile of EBC 8-Isoprostane. Error bars represent standard error of mean. Subjects from the first 8-Isoprostane Quartile had lower mean SGRQ symptom score ( $42.1 \pm 5.3$ ) compared with quartile 4 ( $69.7 \pm 5.3$ ).  $**p=0.0074$  Q1 compared with Q4.



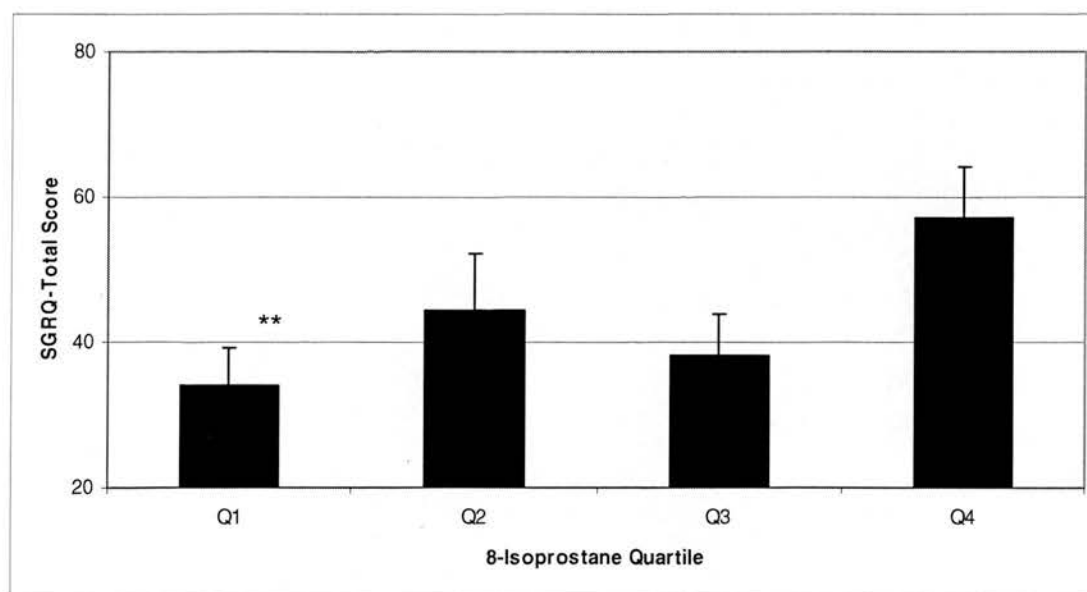
**Figure 4.18 SGRQ Activity Score per 8-Isoprostane Quartile in COPD**

**Ex-smokers** Histograms represent the mean SGRQ activity score per Quartile of EBC 8-Isoprostane. Error bars represent standard error of mean. Subjects from the first 8-Isoprostane Quartile had lower mean SGRQ activity score ( $50.0 \pm 6.4$ ) compared with quartile 4 ( $74.1 \pm 5.3$ ).  $*p=0.0182$  Q1 compared with Q4.



**Figure 4.19 SGRQ Impact Score per 8-Isoprostane Quartile in COPD Ex-smokers**

Histograms represent the mean SGRQ impact score per Quartile of EBC 8-Isoprostane. Error bars represent standard error of mean. Subjects from the first 8-Isoprostane Quartile had lower mean SGRQ impact score ( $21.7 \pm 4.9$ ) compared with quartile 4 ( $43.0 \pm 6.4$ ). \* $p=0.0182$  Q1 compared with Q4.



**Figure 4.20 SGRQ Total Score per 8-Isoprostane Quartile in COPD Ex-smokers**

Histograms represent the mean SGRQ total score per Quartile of EBC 8-Isoprostane. Error bars represent standard error of mean. Subjects from the first 8-Isoprostane Quartile had lower mean SGRQ total score ( $33.9 \pm 4.5$ ) compared with quartile 4 ( $57.0 \pm 6.0$ ). \*\* $p=0.007$  Q1 compared with Q4.

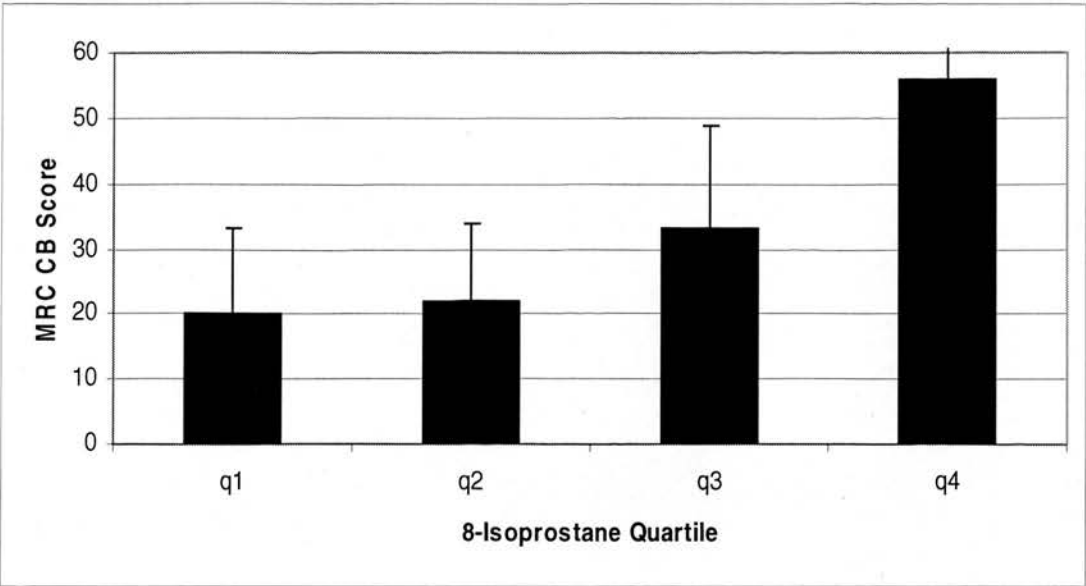
#### 4.6.2 Relationship between 8-Isoprostane in EBC and symptom scores in Ex-smokers with COPD

Mean symptom scores were related to 8-Isoprostane quartiles in EBC. No relationship was demonstrated between levels of oxidative stress in EBC and any of the following; MRC Dyspnoea Score, Wheeze score, the presence of ankle oedema and the presence of nocturnal symptoms. Exacerbation frequency related to 8-Isoprostane quartiles whilst cough, wheeze and chronic bronchitis scores demonstrated trends towards an association with 8-Isoprostane in EBC.

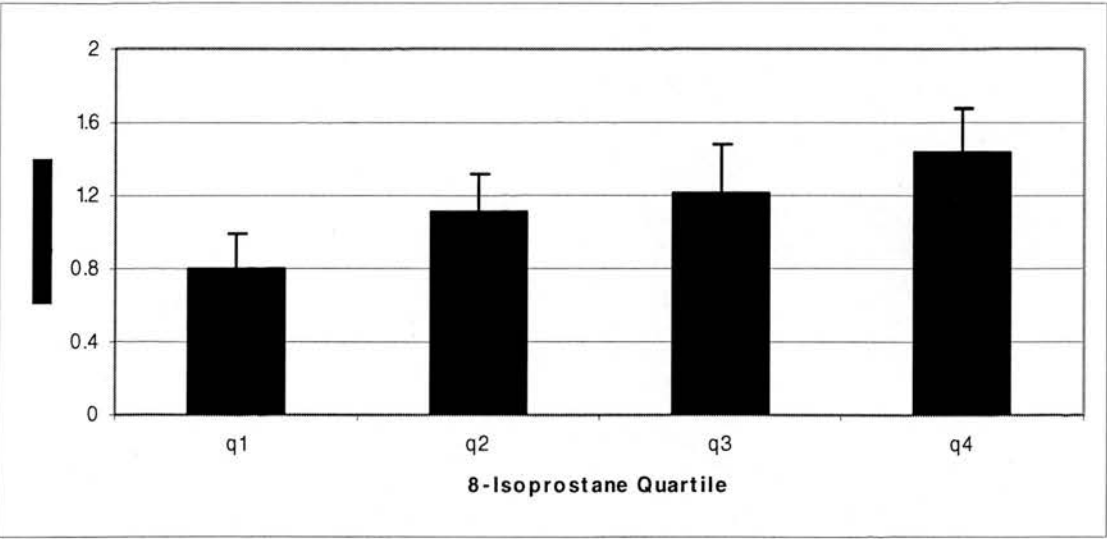
Subjects from the lowest quartile of 8-Isoprostane levels had a significantly lower mean annual exacerbation frequency ( $0.7 \pm 0.3$  exacerbations per year) compared with the highest quartile, ( $3.8 \pm 1.1$  exacerbations per year),  $p=0.03$ . Figure 4.23 Fewer of the subjects in the lowest quartile of measured 8-Isoprostane in EBC reported symptoms of chronic bronchitis (2 out of 10) compared with those in the 4<sup>th</sup> quartile, 5 out of 9. This trend was not statistically significant,  $p=0.10$ . Figure 4.21.

A similar trend was noted between the cough score and 8-Isoprostane levels. Subjects from the 1<sup>st</sup> quartile had a mean cough score of  $0.8 \text{ points} \pm 0.2$  compared with  $1.4 \text{ points} \pm 0.2$  in the 4<sup>th</sup> quartile. Again this trend failed to reach statistical significance,  $p=0.074$ . Figure 4.21a

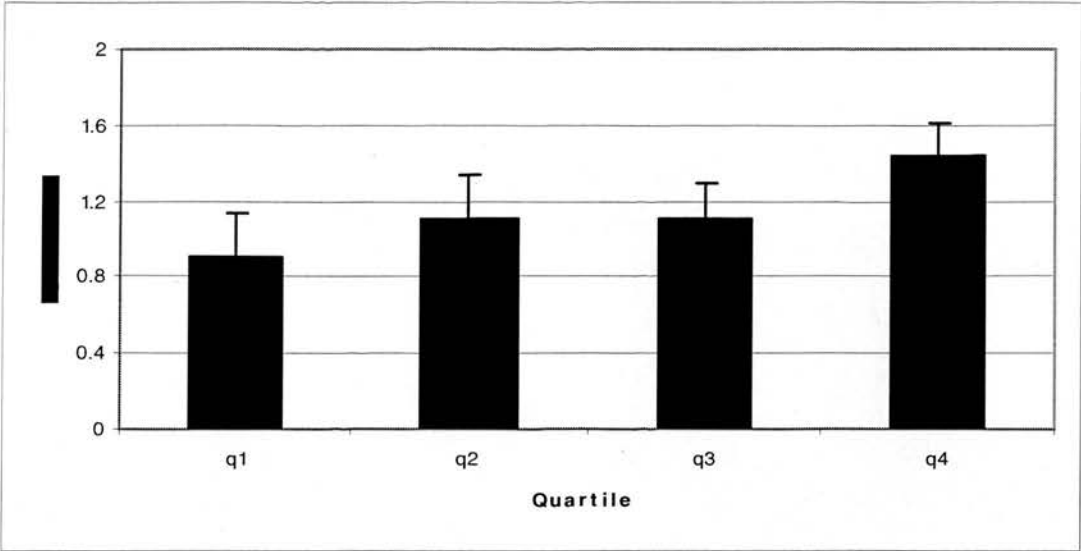
A further trend towards higher reported sputum production scores was noted. The mean score in the highest quartile of the 8-Isoprostane group was  $1.4 \text{ points} \pm 0.2$  compared with the  $0.9 \text{ points} \pm 0.2$  in the lowest quartile. This was not statistically significant,  $p=0.13$ . Figure 4.22. No relationship was demonstrated between any of the symptom scores and levels of pH, PGE<sub>2</sub> and hydrogen peroxide in EBC.



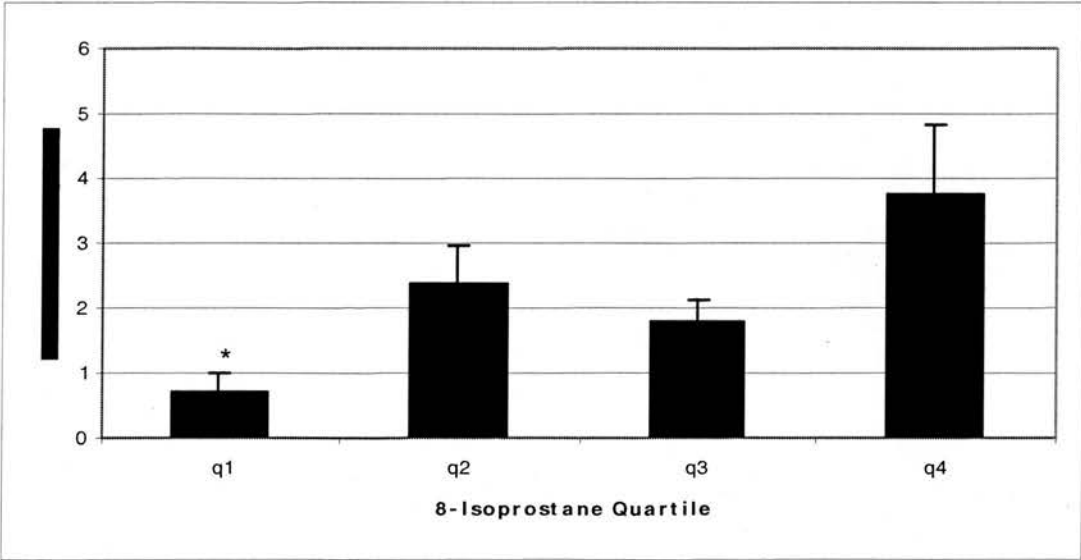
**Figure 4.21 Chronic Bronchitis Score per Quartile EBC 8-Isoprostane in COPD Ex-smokers** Histograms represent the percentage of patients in each EBC 8-Isoprostane Quartile with symptoms of chronic bronchitis. Error bars represent standard error of mean. Trend towards higher percentage of patients with chronic bronchitis in Quartile 4 (56%) vs Quartile 1 (20%)  $p>0.05$



**Figure 4.21a Cough score per Quartile EBC 8-Isoprostane in COPD Ex-smokers** Histograms represent the mean cough score in each quartile EBC 8-Isoprostane. Error bars represent the standard error of mean. Patients in the first quartile had demonstrated a trend towards lower cough scores ( $0.8\pm0.2$ ) when compared with quartile four ( $1.4\pm0.2$ ). This was not quite statistically significant,  $p=0.076$



**Figure 4.22 Sputum Score per EBC 8-Isoprostane Quartile in COPD Ex-smokers** Histograms represent the mean sputum score in each quartile EBC 8-Isoprostane. Error bars represent the standard error of mean. Patients in the first quartile demonstrated a trend towards lower sputum scores ( $0.9 \pm 0.2$ ) when compared with quartile four ( $1.4 \pm 0.2$ ). This was not statistically significant,  $p=0.13$



**Figure 4.23 Annual Exacerbations Frequency per EBC 8-Isoprostane Quartile in COPD Ex-smokers** Histograms represent the mean annual exacerbation frequency in each quartile EBC 8-Isoprostane. Error bars represent the standard error of mean. Patients in the first quartile had lower annual exacerbation frequency ( $0.7 \pm 0.3$ ) when compared with quartile four ( $3.8 \pm 1.1$ ),  $p=0.03$ .

#### 4.6.3 Relationship between 8-Isoprostane and Lung Spirometry in Ex-smokers with COPD

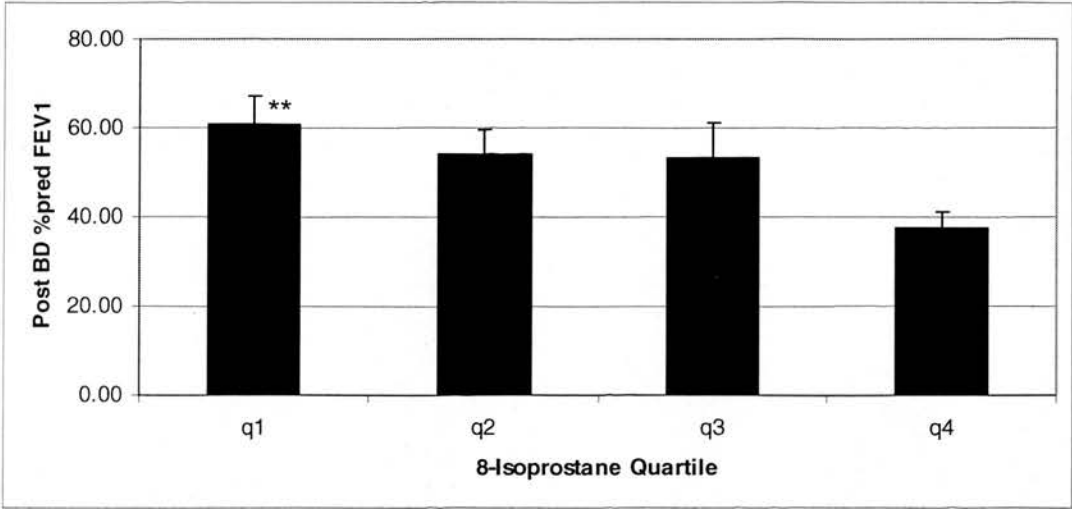
Post bronchodilator FEV<sub>1</sub>, FVC, FEV<sub>1</sub>/FVC ratio and the degree of reversibility were compared with the 8-Isoprostane levels in EBC. Both absolute lung volumes in litres and percentage predicted values were analysed. The ex-smoking COPD group was divided into quartiles dependant on the level of 8-Isoprostane levels. Mean values for the lung function parameters mentioned above were then calculated and between quartile analyses performed.

The highest quartile of 8-Isoprostane levels in EBC had a significantly lower mean percentage predicted FEV<sub>1</sub> post bronchodilator ( $37.6\% \pm 3.5$ ) compared with the lowest quartile of 8-Isoprostane ( $60.7\% \pm 6.5$ ). This difference was statistically significant,  $p=0.009$ . Figure 4.24

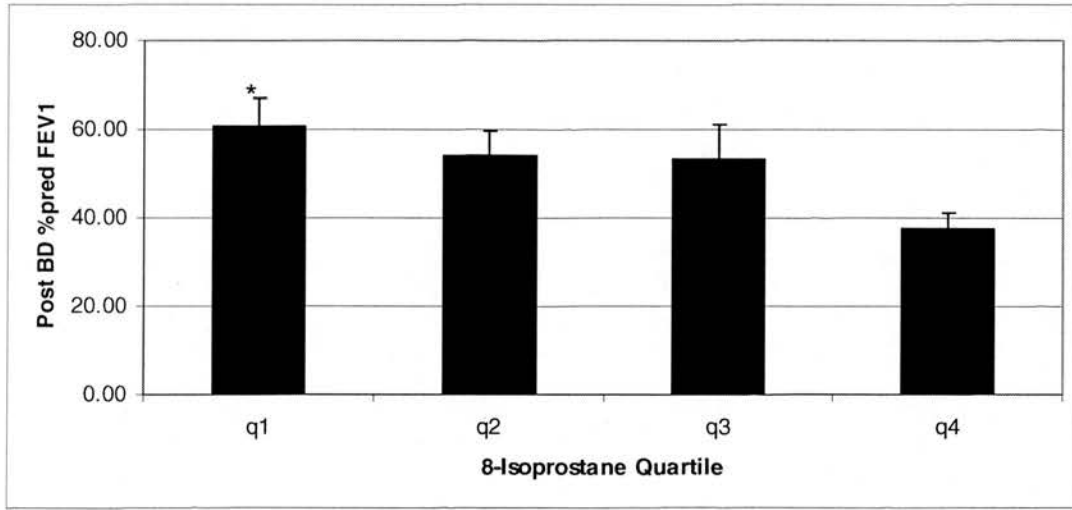
The mean ratio of FEV<sub>1</sub>/FVC was also significantly lower in subjects from the quartile with the highest levels of 8-Isoprostane in EBC,  $39.1\% \pm 3.0$ , compared with the lowest quartile group with mean levels of  $54.7\% \pm 4.7$ .  $p=0.017$ . Figure 4.25

No significant difference was demonstrated between 8-Isoprostane quartiles and any absolute dynamic lung measurement. The degree of reversibility and the percentage predicted FVC did not relate to EBC measurement of 8-Isoprostane.





**Figure 4.24** Percentage Predicted FEV<sub>1</sub> post Bronchodilator per EBC 8-Isoprostane Quartile Histograms represent the mean percentage predicted FEV<sub>1</sub> measured post bronchodilator in each quartile of 8-Isoprostane measurement in EBC. The error bars represent the standard error of mean. Subjects in the lowest quartile of EBC 8-Isoprostane had statistically significant higher FEV<sub>1</sub> (60.7%±6.5) when compared with Quartile 4 (37.6%±3.5), \*\*p=0.009.



**Figure 4.25** Ratio of FEV<sub>1</sub>/FVC post bronchodilator per EBC 8-Isoprostane Quartile in COPD Ex-smokers. Histograms represent the mean FEV<sub>1</sub>/FVC ratio measured post bronchodilator in each quartile of 8-Isoprostane measurement in EBC. The error bars represent the standard error of mean. Subjects in the lowest quartile of EBC 8-Isoprostane had statistically significant higher FEV<sub>1</sub>/FVC ratio (54.7%±4.7) when compared with Quartile 4 (39.1%±3.0), \*p=0.017.

#### 4.6.4 Relationship between Hydrogen Peroxide in EBC and Lung Spirometry in Ex-smokers with COPD

Hydrogen peroxide levels in Exhaled Breath Condensate were divided into quartiles. The mean levels of the dynamic lung function tests mentioned in the previous section were then calculated for each quartile and between groups comparisons were made.

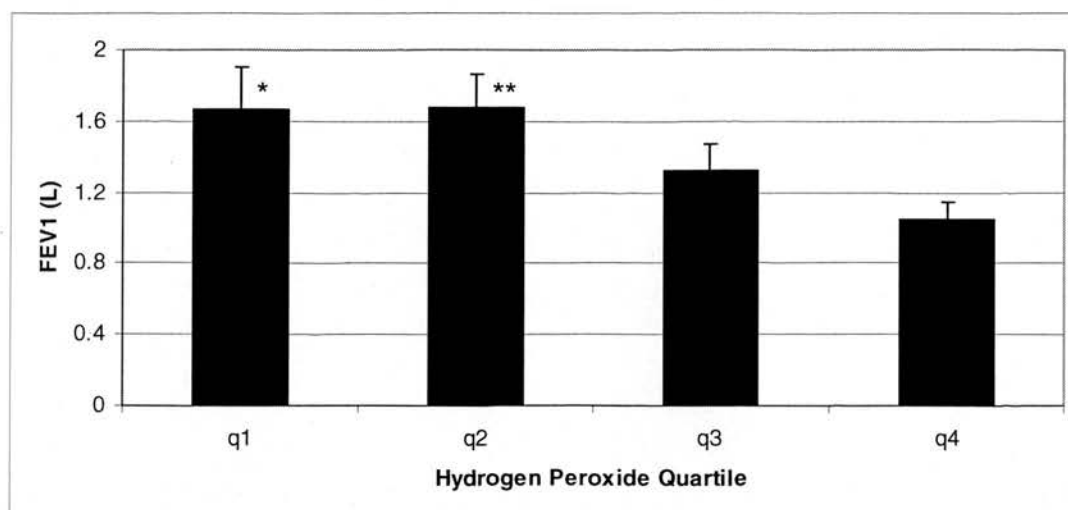
The mean absolute FEV<sub>1</sub> post bronchodilator was lower in the 4<sup>th</sup> quartile of hydrogen peroxide EBC (1.05 L  $\pm$  0.1) compared with both the first quartile (1.67 L  $\pm$  0.23,  $p=0.046$ ) and the second quartile (1.68 L  $\pm$  0.19,  $p=0.001$ ). Figure 4.26.

The absolute levels of Forced Vital Capacity post bronchodilator were also associated with hydrogen peroxide levels. Subjects with the highest levels of H<sub>2</sub>O<sub>2</sub> had significantly lower mean FVC (2.4 L  $\pm$  0.19) compared with those from the lowest quartile (3.34 L  $\pm$  0.32),  $p=0.02$ . Mean FVC levels in the second quartile (3.2 L  $\pm$  0.32) were close to statistical significance compared with 4<sup>th</sup> quartile,  $p=0.053$ .

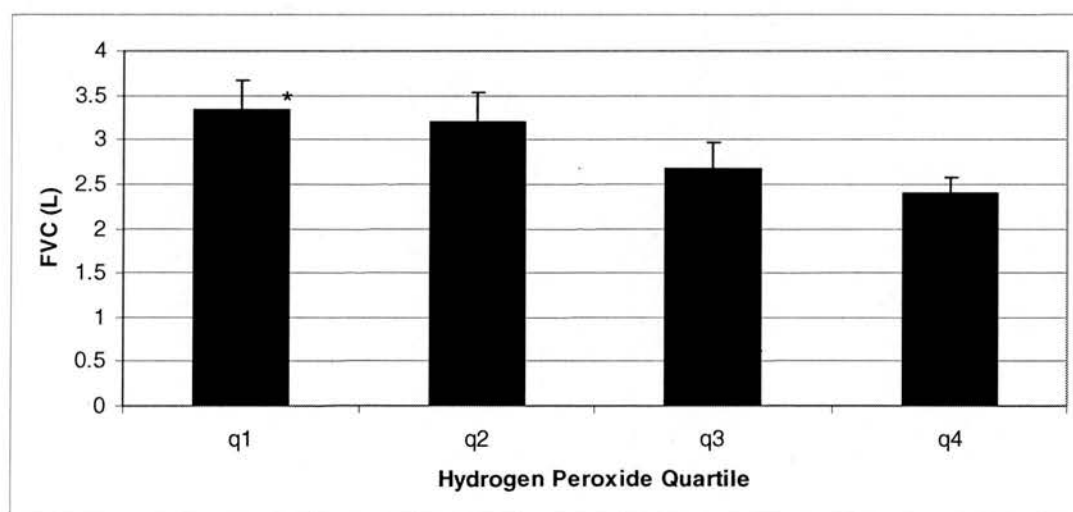
Figure 4.27

Percentage predicted FEV<sub>1</sub> post bronchodilator was also associated with hydrogen peroxide levels. Subjects with the highest levels of hydrogen peroxide had lower mean FEV<sub>1</sub> (39.7%  $\pm$  4.2) compared with subjects from the second quartile (66.0%  $\pm$  6.3),  $p=0.003$ , whilst subjects from the lowest quartile had a trend towards higher mean FEV<sub>1</sub> post BD (54.3%  $\pm$  6.3) compared with the 4<sup>th</sup> quartile,  $p=0.068$ . Figure 4.28. There was no relationship between reversibility to salbutamol or post bronchodilator percentage predicted FVC and hydrogen peroxide levels.

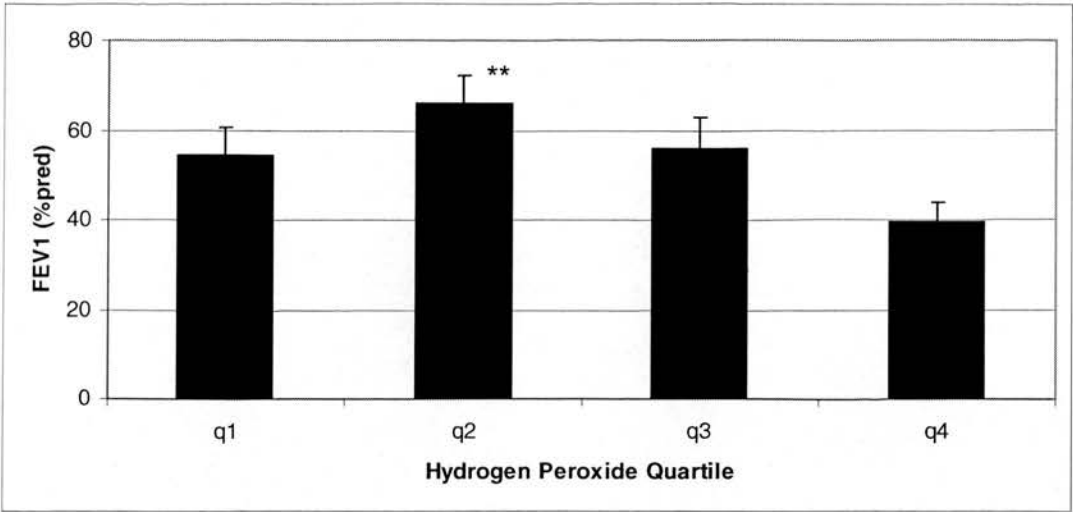
Neither PGE<sub>2</sub> nor pH levels in EBC appeared to relate to any of the lung function measurements.



**Figure 4.26 Post Bronchodilator FEV<sub>1</sub> (L) per Hydrogen Peroxide Quartile in COPD Ex-smokers** Histograms represent the mean FEV<sub>1</sub> (L) post bronchodilator in each of the quartiles of hydrogen peroxide EBC levels. Error bars represent standard error of mean. Subjects from the first quartile and second quartiles of hydrogen peroxide EBC levels had higher mean FEV<sub>1</sub> ( $1.67 \pm 0.23$  and  $1.68 \pm 0.19$  respectively) when compared with quartile four ( $1.05 \pm 0.1$ ), \* $p=0.046$  Q1 vs Q4, \*\* $p=0.001$  Q2 vs Q4



**Figure 4.27 Post Bronchodilator FVC (L) per Hydrogen Peroxide Quartile in COPD Ex-smokers.** Histograms represent the mean FVC (L) post bronchodilator in each of the quartiles of hydrogen peroxide EBC levels. Error bars represent standard error of mean. Subjects from the first quartile of hydrogen peroxide EBC levels had a higher mean FVC ( $3.34 \pm 0.32$ ) when compared with quartile four ( $2.40 \pm 0.19$ ), \* $p=0.02$  Q1 vs Q4. Mean FVC levels ( $3.20 \pm 0.33$ ) in the second quartile was close to a statistically significant difference when compared with quartile 4,  $p=0.053$ .



**Figure 4.28** Post Bronchodilator Percentage Predicted FEV<sub>1</sub> per Hydrogen Peroxide Quartile in COPD Ex-smokers. Histograms represent the mean percentage predicted FEV<sub>1</sub> post bronchodilator in each of the quartiles of hydrogen peroxide EBC levels. Error bars represent standard error of mean. Subjects from the second quartile of hydrogen peroxide EBC levels had a higher mean %pred FEV<sub>1</sub> ( $66.0 \pm 6.3$ ) when compared with quartile four ( $39.7 \pm 4.2$ ),  $**p=0.003$  Q2 vs Q4. Mean %pred FEV<sub>1</sub> levels ( $54.3 \pm 6.3$ ) in the first quartile was close to a statistically significant difference when compared with quartile 4,  $p=0.068$ .

## 4.6 Conclusions

The study populations were not age matched. Prostaglandin E2 and pH were measurable in the Exhaled Breath Condensate of subjects with COPD and healthy controls. 8-Isoprostane and hydrogen peroxide were also detectable in the majority of trial subjects. However these measurements were often close to the limit of detection of the assay.

There was no major variation in the pH of breath condensate or levels of 8-Isoprostane found in breath condensate between patients with COPD and controls without respiratory disease. Furthermore current smoking habit did not appear to affect levels of these two biomarkers.

Ex-smokers, both subjects with COPD and controls, had higher mean levels of hydrogen peroxide in EBC compared with control non-smokers and current smokers. Subjects with COPD had higher mean levels of hydrogen peroxide compared with controls. The majority of subjects in the control group had significant non respiratory co-morbidities. Subjects with co-morbidity appeared to have elevated levels of PGE2 and 8-Isoprostane when compared with controls with no co-morbidities. This observation is made without fully categorising the non respiratory co-morbidities. No similar trend was demonstrated in the pH level or hydrogen peroxide measurements in EBC. Levels of PGE2 and 8-Isoprostane were similar in the group with COPD compared with the group with non respiratory co-morbidity.

Levels of the four biomarkers in EBC did not appear to relate to age, sex, nutritional status or lung function in the control subjects. In a similar fashion, PGE2

and pH in EBC in the COPD group bore no relation with any of the identified parameters of disease.

In the group of COPD current smokers a relationship was demonstrated between levels of 8-Isoprostane and ICS use, with a trend towards a similar result in PGE<sub>2</sub>. No other relationship was identified with any of the health parameters applied. In contrast, 8-Isoprostane levels in the EBC of ex-smokers with COPD were significantly related to health status, with higher levels associated with worsening health status, exacerbation frequency and symptom scores. Higher 8-Isoprostane levels were also related to a reduced FEV<sub>1</sub>. Hydrogen peroxide levels also appeared to be inversely correlated with airflow limitation.

In conclusion, none of the four biomarkers studied appear to be useful in differentiation of COPD from patients without respiratory disease. Non respiratory co-morbidity appears to affect measurement of inflammation and oxidative stress in EBC.

EBC measurement in current smokers with COPD did not appear to help characterise the disease process. In contrast, in the group of subjects with COPD who were ex-smokers, 8-Isoprostane levels, and to a lesser extent hydrogen peroxide, were related to a variety of health parameters. The number of subjects in each quartile of the two COPD groups was small.

The positive results demonstrated in section 6.4 should therefore be interpreted with caution. A repeat study, with a larger sample size, would be required to support these initial results.

Assessment of EBC Oxidative Stress in subjects with COPD who are not currently smoking may be of use in classifying the extent of ongoing disease activity. Longitudinal follow-up of these subjects may provide further intervention regarding this.

## **Chapter 5**

# **Impact of Inhaled Nacystelyn on Patients with Chronic Obstructive Pulmonary Disease**



## **Chapter 5    Impact of Inhaled Nacystelyn on Patients with Chronic Obstructive Pulmonary Disease**

### **5.1    Introduction**

Chapter One of this thesis describes the oxidative stress hypothesis for Chronic Obstructive Pulmonary Disease. In this hypothesis, subjects with COPD have higher levels of oxidative stress in the lungs along with an impaired anti-oxidant defence system. This results in direct damage to the lung together with increased inflammation and cellular influx. These processes result in further tissue damage and the development of COPD.

The major extracellular anti-oxidant in the lung is glutathione. Glutathione and cysteine, the immediate precursor of glutathione, have poor bio-availability, limiting their clinical usefulness as a therapy. N-Acetylcysteine is a precursor of cysteine. When administered orally it is readily metabolised by the liver to produce glutathione. The majority of this glutathione produced is consumed in the liver, limiting the effect it will have on glutathione levels in the lung. N-Acetylcysteine is acidic and not tolerated when inhaled.

Nacystelyn is the lyseine salt of N-Acetylcysteine, has a neutral pH and is tolerated by inhalation. Inhalation of Nacystelyn (NAL) may increase the levels of glutathione in the lung. As discussed in chapter 1, improved pulmonary anti-oxidant defences may reduce the level of oxidative stress in subjects with COPD. Reduced oxidative stress may reduce inflammation; improve health status and lung function in these patients.

## 5.2 Study Hypothesis

**Inhaled antioxidant therapy will improve health status, lung function and biomarkers of inflammation and oxidative stress in patients with COPD**

## 5.3 Study Objectives

The primary objective was to determine if the efficacy of NAL 20 mg bd and NAL 40 mg bd was superior to that of placebo in a 2 centre, randomised, double-blind, placebo-controlled, Phase IIa study of patients with moderate to severe COPD after 12 weeks administration. Health status measured using the St. George's Respiratory Questionnaire (SGRQ) was the primary endpoint (appendix 5.3).

Secondary objectives were to evaluate the efficacy of NAL 20 mg bd and NAL 40 mg bd compared with that of placebo using a variety of parameters, including non-invasive biomarkers of oxidative stress and inflammation, diary card recordings and lung function testing.

## **5.4 Study Protocol**

### **5.4.1 Introduction**

Subjects were recruited after clinical and spirometric confirmation of their diagnosis. An initial two to four week run-in period was followed by twelve weeks of treatment of one of the three arms of the study, randomised in a double blind manner on a 1:1:1 ratio. Subjects were assessed during the study, immediately at the end of trial and two weeks after trial completion.

### **5.4.2 Trial Subject Selection**

Informed consent was obtained prior to assessing subjects' suitability for trial participation. Subjects with a firm clinical diagnosis of COPD were recruited into the trial. Confirmation of the diagnosis was made from clinical history, physical examination, spirometric assessment and review of case records.

To participate in the study subjects were required to be over 40 years of age, be either current or ex-smokers with at least 10 pack years and have a confirmed clinical diagnosis of COPD. Subjects were also required to have spirometric confirmation of irreversible airflow limitation, have chronic symptoms of breathlessness and have had at least one exacerbation of their condition in the last year. Subjects were required to be clinically stable, as defined by no exacerbation in the previous month. (appendix 5.2)

The population studied is prone to exacerbations; one such exacerbation requiring steroids and/or antibiotics during the trial would not result in withdrawal from the trial. Failure to recover from this exacerbation or a subsequent exacerbation during

the trial period would indicate that the subject's disease was too unstable to allow continued trial participation.

Subjects were excluded from the trial if they had a clinical diagnosis of asthma or any other active lung diseases. Subjects with unstable non-respiratory conditions or a history of malignancy in the previous five years were also excluded. (appendix 5.2)

#### **5.4.3 Spirometric Assessment**

Clinical suspicion of COPD was confirmed by spirometric testing. As mentioned in the GOLD definition, subjects with COPD demonstrate airflow limitation which is not fully reversible. For the purpose of this study we recruited patients who demonstrated moderate to severe airflow limitation defined as a Forced Expiratory Volume<sub>1</sub> (FEV<sub>1</sub>) between 30-70% predicted with a FEV<sub>1</sub> to Forced Vital Capacity (FVC) ratio of less than 70%. Reversibility of airflow obstruction was then assessed twenty minutes after administration of 2.5mg nebulised salbutamol. Significant reversibility was defined as greater than 200ml increase absolute FEV<sub>1</sub> and greater than 15% increase percent predicted FEV<sub>1</sub>. Subjects who demonstrated this degree of reversibility were excluded from the trial (appendix 2.5).

#### 5.4.4 Therapy during Trial

Administration of medications was assessed prior to trial participation and monitored for the duration of the trial. Attempts were made to minimise alterations to medications during the course of the trial. No alterations were made to non-respiratory medications.

Inhaled  $\beta_2$ -agonists, both short-acting (e.g. salbutamol) and long-acting (e.g. salmeterol) were allowed during the study as were inhaled short acting anticholinergics (e.g. ipratropium bromide). Inhaled long acting anticholinergics (e.g. tiotropium) were not permitted during the trial; subjects on this medication were excluded from trial entry. Oral mucolytics and theophyllines were also prohibited during the trial and subjects on these medications were excluded from trial entry.

Inhaled corticosteroids (ICS) are widely used in the management of COPD. To ensure that administration of ICS did not lead to bias in the trial it was decided to ensure that all patients were on regular ICS for a minimum of 4 weeks prior to trial entry, and that all subjects were taking the same type, dose and device of ICS.

Subjects who were not already on an ICS were commenced on Fluticansone Accuhaler 250  $\mu$ g bd 4 weeks prior to randomisation. Those already on an ICS were changed to Fluticansone Accuhaler 250  $\mu$ g bd. Subjects on other ICS therapy were asked to discontinue this therapy. Subjects on combination ICS and long acting  $\beta$  agonist therapy were asked to discontinue this medication and were

issued with an appropriate long acting  $\beta$  agonist along with the standard ICS used in this study. Subjects using different delivery systems of Fluticasone were asked to discontinue these and take an accuhaler.

Administration of one course of oral corticosteroids for an exacerbation of COPD was permitted at a dose of 30 mg prednisolone per day for a maximum of two weeks. Antibiotics were allowed if given for less than 2 weeks during a single respiratory exacerbation or for reasons other than a respiratory exacerbation.

#### **5.4.5 Screening Demographics prior to trial entry**

The following screening demographic information was obtained; age, sex, race, weight, height, body mass index, alcohol intake, smoking status and smoking pack years.

All relevant medical history was recorded. Active medical history, defined as medical conditions that were ongoing at start of study, was to be summarised separately.

#### 5.4.6 Trial Subject Participation

The trial consisted of a 2-4 week run-in period, a 12 week treatment period and a 2 week follow up period with a total of 6 study visits. (appendix 5.1)

##### 5.4.6.1 Run-In Period Visit 1 (Week -4 to -2)

After consent was confirmed, clinical assessment including spirometry was performed. Each patient was then instructed how to use the study medication inhaler device (appendix 5.5). Patients were issued with placebo capsules, 50mg anhydrous  $\beta$ -lactose 4 capsules bd, to be taken along with Fluticasone Accuhaler 250  $\mu$ g bd.

Subjects were issued with a diary card (appendix 5.4) and asked subjectively to record daily their symptoms of cough, wheeze, breathlessness, sputum production and colour, nocturnal symptoms along with the impact of disease on activities of daily living. Subjects were also encouraged to record any new or changing symptoms which they experienced during the duration of the trial.

Plasma sampling was performed; measurement was made of renal and liver function including glucose, creatinine, total protein, alanine aminotransferase, aspartate aminotransferase, gamma-glutamyl transferase, total bilirubin, uric acid, alkaline phosphatase, sodium, potassium, phosphate, albumin and calcium. Full blood count was also measured including haemoglobin concentration, haematocrit, platelet, red blood cell count, mean cell volume, white blood cell and differential counts. Samples were also taken to allow measurement of systemic oxidative stress and inflammation (appendix 2.4)

Subjects were then asked to return after 2 weeks if they were currently on ICS and after 4 weeks if they were steroid naïve.

#### **5.4.6.2 Randomisation - Visit 2 (Week 0)**

At Visit 2 (Week 0), subject's willingness to participate in the trial was confirmed.

Repeat clinical status was assessed; any significant change from previous attendance was recorded. Health status was measured using St Georges Respiratory Health Questionnaire (SGRQ). Diary cards were reviewed and medication returned, capsules counted to ensure compliance, consumption of 80% of study medication considered to be good compliance.

Exhaled breath condensate was obtained, subjects were asked to breathe for ten minutes into a Jaeger Ecosystem, exhaled breath condensate was then aliquoted and frozen at -80°C (appendix 2.1). Plasma sampling was performed followed by spirometry pre and post nebulised 2.5mg salbutamol. Finally sputum induction was carried out, samples obtained were then processed within 4 hours of collection (appendix 2.3). Subjects were then randomised and issued with study medication along with a fresh diary card.

Patients were randomised in a 1:1:1 ratio to receive NAL 20 mg bd, NAL 40 mg bd or placebo along with Fluticasone. Randomisation was performed using a validated system that automates the random assignment of treatment groups to randomisation numbers. The randomisation scheme was held by authorised personnel not actively involved in the study and locked by them after approval.



Selection And Timing Of Dose For Each Patient

Group	NAL 20 mg bd	NAL 40 mg bd	Placebo
Morning	2 Capsules: NAL 10 mg 2 Capsules: NAL Placebo  1 <u>Blister</u> : Fluticasone 250 mcg	4 Capsules: NAL 10 mg  1 <u>Blister</u> : Fluticasone 250 mcg	4 Capsules: NAL Placebo  1 <u>Blister</u> : Fluticasone 250 mcg
Evening	2 Capsules: NAL 10 mg 2 Capsules: NAL Placebo  1 <u>Blister</u> : Fluticasone 250 mcg	4 Capsules: NAL 10 mg  1 <u>Blister</u> : Fluticasone 250 mcg	4 Capsules: NAL Placebo  1 <u>Blister</u> : Fluticasone 250 mcg

Doses were to be taken twice daily, administered using the MIAT monodose DPI, allowing the powder to be inhaled through the mouth. The 20 mg dose group took two active nacystelyn capsules and two placebo capsules, the 40 mg dose group took four active nacystelyn capsules, and the placebo group took four placebo capsules in the morning and in the evening. Dosing was to be 12 hours apart.

In addition to their study medication all patients were to take a fixed daily dose of inhaled fluticasone (250 µg bd) to standardise the inhaled corticosteriod treatment for all patients.

#### **5.4.6.3 Trial Monitoring Visit 3 (Week 2)**

At Visit 3, clinical assessment including spirometry was performed. Any significant changes in symptoms were recorded. Compliance with therapy was assessed.

Diary cards were reviewed and a new diary card was issued.

#### **5.4.6.4 Trial Monitoring Visits 4 (Week 4) and 5 (Week 12)**

At Visit 4 (Week 4) and Visit 5 (Week 12), clinical assessment including spirometry was performed, along with review of diary cards. Any significant change in symptoms or new symptoms was recorded. Health status was again assessed using SGRQ. Treatment compliance was confirmed by counting medications returned. EBC, plasma and induced sputum were obtained and stored for analysis.

At Visit 4 subjects were issued with further medication and diary cards. At Visit 5 all medication was collected and it was ensured that subjects had a supply of their previous inhaled therapy, at which stage trial participation was ceased.

#### **5.4.6.5 Trial Follow-Up - Visit 6 (Week 14)**

At Visit 6, clinical assessment including spirometry was performed and any change in symptoms recorded.

## **5.5 Efficacy Analysis**

Measurement of health status was assessed using health questionnaires and symptom diary cards. Objective analysis of response to therapy was carried out using spirometric measurement of lung function along with non-invasive biomarkers from exhaled breath condensate, induced sputum and plasma.

### **5.5.1 St Georges Respiratory Health Questionnaire (appendix 5.3)**

Initially published in 1991 the St Georges Respiratory Health Questionnaire is a well validated assessment of health status in COPD. Subjects are asked to respond to 72 questions. The responses are then applied to a formula which generates a total score. The scores range from 0, no effect on health status, to 100, maximal effect. This total score can then be subdivided into three component scores (symptoms, activities and impact) each of which again range from 0-100. An improvement of 4 units has been shown to be associated with a clinically significant improvement(389).

### **5.5.2 Diary Cards (appendix 5.4)**

Subjects were asked to keep a daily diary. They were asked to record scores for cough (0=no cough to 3=cough throughout day), breathlessness (0=no breathlessness to 4=breathlessness at rest), sputum production (0=no sputum to 3=sputum throughout day), sputum colour (0=no sputum to 4 dark green/ yellow sputum), number of night awakenings, time off work, time unable to carry out household tasks and any changes in medications. They were also encouraged to record any new or changing symptoms which they experienced during the course of the trial.

### 5.5.3 Spirometry (appendix 2.5)

Lung function was assessed using a Vitalograph Spirometer. Subjects were requested to abstain from short acting  $\beta$ -agonists and anticholinergics for 8 hours and long acting  $\beta$ -agonists for 24 hours.

Subjects were requested to inhale fully then exhale as hard and fast as possible into a mouthpiece until their lungs emptied. Each test was repeated until three recordings had been obtained with a variation of less than 5%

Subjects were then given 2.5mg of nebulised salbutamol and lung function was repeated fifteen minutes later.

Percentage predicted lung volumes, dependant on age, height, sex and race were calculated using the following formula:-

$$(4.3 \times \text{height}) - (0.029 \times \text{age}) - 2.49 \quad \text{for males}$$

$$(3.95 \times \text{height}) - (0.025 \times \text{age}) - 2.60 \quad \text{for females}$$

### 5.5.4 Non Invasive Biomarkers

The utility of biomarkers of airways inflammation and oxidative stress is discussed in Chapter 1 of this thesis. The following biomarkers were selected for measurement during this trial on the basis of previous published studies. Exhaled Breath Condensate was collected using a standard operating protocol (appendix 2.1) and levels of Hydrogen Peroxide, 8-Isoprostane, Leukotriene B4 and Nitrotyrosine measured (appendix 2.6, 2.7,2.8). Induced Sputum was collected (appendix 2.2, 2.3), differential cell counts calculated and supernatant analysed for levels of IL-1 $\beta$ , IL-6, IL-8 and TNF- $\alpha$ (appendix 2.9).

Plasma samples were collected and differential cell counts, high sensitivity CRP and Thiobarbituric acid reactive substances measured (appendix 2.10, 2.11).

## **5.6 Ethical Approval**

Ethical permission for this study was obtained from the Ethics Committee of Lothian Acute Hospitals NHS Trust prior to study commencement. Written information regarding this study was provided to all subjects at least 24 hours prior to trial recruitment. Written informed consent was signed before study participation

## **5.7 Statistical Analysis**

Analysis of the baseline characteristics of the study groups was performed using Tukey One Way Analysis of Variance (ANOVA), Fisher's Exact Test and Non Paired T-Tests. Exacerbations rates and side effects during the trial were compared using Fisher's Exact Test and non-paired T-test. Spirometric data, which had a parametric distribution, was analysed using Tukey One Way Analysis of Variance (ANOVA). Within-group analysis comparing changes from baseline to a study visit was performed using paired t-test.

Data obtained from the St Georges Health Questionnaire, diary cards and non-invasive biomarker measurement was of a non-parametric distribution. Group analysis was therefore performed using the Dunn version of ANOVA. Comparison within groups of two points in the trial was performed using Wilcoxon matched pairs.

This study was conducted over an 18 month time period. A power calculation prior to trial commencing indicated that in the region of 100 patients would be required in each study arm sufficiently to power the SGRQ results in the study, 300 patients in total. It was understood prior to trial commencement that we would be unlikely to recruit this number of patients. This was the first clinical study of this therapy in COPD. The study was run as a "proof of principle" and to observe tolerance of the

treatment with the appreciation that it may be insufficiently powered to answer the primary endpoint of health status change.

## 5.8 Demographics

A total of 58 patients participated in the trial. There were 19, 20 and 19 patients in the 40mg bd, 20mg bd and placebo groups respectively. The patients were predominately males (36M, 22F) with no significant variation between the three arms. Mean age was 66.5 ( $\pm 8.8$ ), range 48-94, BMI 26.1 ( $\pm 5.2$ ) range 15.8-41.4, neither of these parameters varied significantly between the treatment groups. (Table 5.1)

Twenty-six of the 58 (45%) patients were current smokers. The placebo (12/19) and 40mg bd (9/19) groups had more current smokers than the 20mg bd group (5/20), although these differences were not statistically significant ( $p > 0.05$ ). Average smoking history was 47( $\pm 21.5$ ) pack years, range 15-120 pack years. There were no statistically significant differences in pack years between the groups.

Thirty-eight of the 58 (66%) patients participating in the trial were on inhaled corticosteroids prior to trial entry. Sixteen of the 20 (80%) patients in the 20mg bd, 13 of the 19 (68%) in the placebo group and 9 of the 19 (47%) in the 40mg bd having recently been on ICS. 59% of patients were on long acting bronchodilators prior to trial entry.

Trial subjects' health status, as assessed by St Georges Respiratory Questionnaire, demonstrated wide variation between individuals, but did not differ significantly between the three treatment arms prior to trial entry. The SGRQ will be discussed in more detail in the results section.

Lung function prior to study commencement demonstrated moderate to severe airflow limitation, mean absolute FEV<sub>1</sub> post bronchodilator 1.30l (SD 0.46l), range 0.42-2.84l, mean percentage predicted FEV<sub>1</sub> post bronchodilator 49% predicted (SD 12.1%), range 28-77%.



Group	Placebo	20mg NAL bd	40mg NAL bd
Number	19	20	19
Age mean	65.7	67.3	67.9
Range	(48-79)	(50-94)	(50-82)
Sex (m/f)	11/8	13/7	12/7
BMI mean	26.6	25.3	26.4
Range	(15.8-36.7)	(18.3-36.5)	(17.1-41.4)
Smoking Status (current/ex)	12/7	5/15	9/10
Pack Years mean	46.6	37.7	57.2
Range	(24-81)	(15-65)	(25-120)
ICS	13/20	16/20	9/19
LABD	10/19	14/20	10/19
FEV <sub>1</sub> (l) mean	1.36	1.23	1.29
Range	(0.55-2.84)	(0.82-1.99)	(0.42-2.55)
FEV <sub>1</sub> (%) mean	50(34-74)	47(30-77)	47(27-72)
Range			
SGRQ total mean	55.8	55.4	49.7
Range	(36.4-82.7)	(23.3-87.0)	(22.8-80.4)

**Table 5.1 Patient demographics prior to trial commencement per treatment group.** BMI=body mass index, ICS=inhaled corticosteroids, LABD=long acting bronchodilator, FEV<sub>1</sub>= forced expiratory volume in 1 second post bronchodilator, SGRQ=St Georges Respiratory Questionnaire.

## 5.9 Study Details

The study ran from the 6<sup>th</sup> of January 2003 until the 18<sup>th</sup> of August 2004. A total of 58 patients were randomised into the study. Overall 44 of the 58 (74.1%) completed the study. Fourteen patients (24.1%) did not complete the study. Only one clinically significant exacerbation of COPD defined, according to ERS/ATS guidelines, as “an acute change in a patient’s baseline breathlessness, cough and/or sputum beyond day-to-day variability sufficient to warrant a change in therapy”, was allowed during the trial. As mentioned in section 5.4.2, the trial protocol stated that failure to recover from the first exacerbation or a further exacerbation would be felt to indicate clinically unstable disease and would result in withdrawal from the trial. Two subjects (1 in the 40mg bd NAL, 1 in the 20mg bd NAL) were withdrawn from the study due to recurrent exacerbations of COPD.

Eleven of these 14 patients withdrew because of side effects. None of these side effects were classified as major eg. life threatening or requiring hospitalisation, urgent medical review or change in medication. Two subjects withdrew solely due to worsening respiratory symptoms other than recurrent exacerbations of COPD. Four withdrew because of a combination of side effects from respiratory and other systems. Five withdrew because of symptoms not originating from the lower respiratory tract. Other systems involved included neurological (3), cardiovascular (2), musculoskeletal (2), genitourinary (1) and dermatological (1). There was no significant difference in the number of subjects withdrawing from the trial due to

side effects between the three treatment arms. One subject in the 40mg bd NAL withdrew consent without experiencing side effects.

	Placebo n (%)	NAL 20mg bd n (%)	NAL 40mg bd n (%)
Randomised	19 (100)	20 (100)	19 (100)
Completed	15 (79)	16 (80)	13 (68)
Withdrawn	4 (21)	4 (20)	6 (32)
Adverse Event	4 (21)	3 (15)	4 (21)
Recurrent Exac	0 (0)	1 (5)	1 (5)
Withdrew Consent	0 (0)	0 (0)	1 (5)

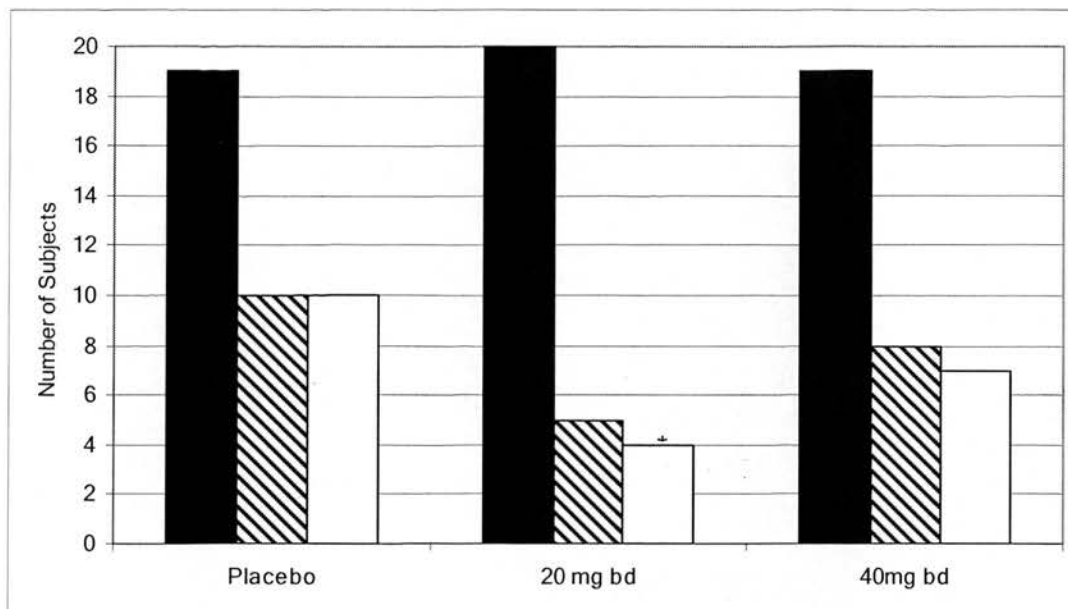
Table 5.2 Subject trial completion per treatment arm and reason for withdrawal.

## 5.10 Side Effects and Adverse Events

A total of 155 side effects were reported during the course of the study. Side effects which occurred frequently (>5% of side effects in study population) within the groups are described below.

The most frequent adverse event in each treatment group was an exacerbation of COPD. A total of 23 exacerbations affected 21 subjects participating in the trial. Fewer patients had exacerbations in the 20mg bd NAL group compared with placebo ( $p=0.048$ ). No statistical difference was seen between the 40mg bd NAL group and placebo or between the 20mg bd NAL group and the 40mg bd NAL group. Figure 5.1.

Side effects other than exacerbations of COPD were frequently reported during the trial. Subjects in the 40mg bd NAL and 20mg bd NAL were more likely to report symptoms of sore throat or cough compared with placebo, and less likely to report symptoms of headache, muscle cramp, upper respiratory tract infection or dry mouth. None of the differences between groups was statistically significant.

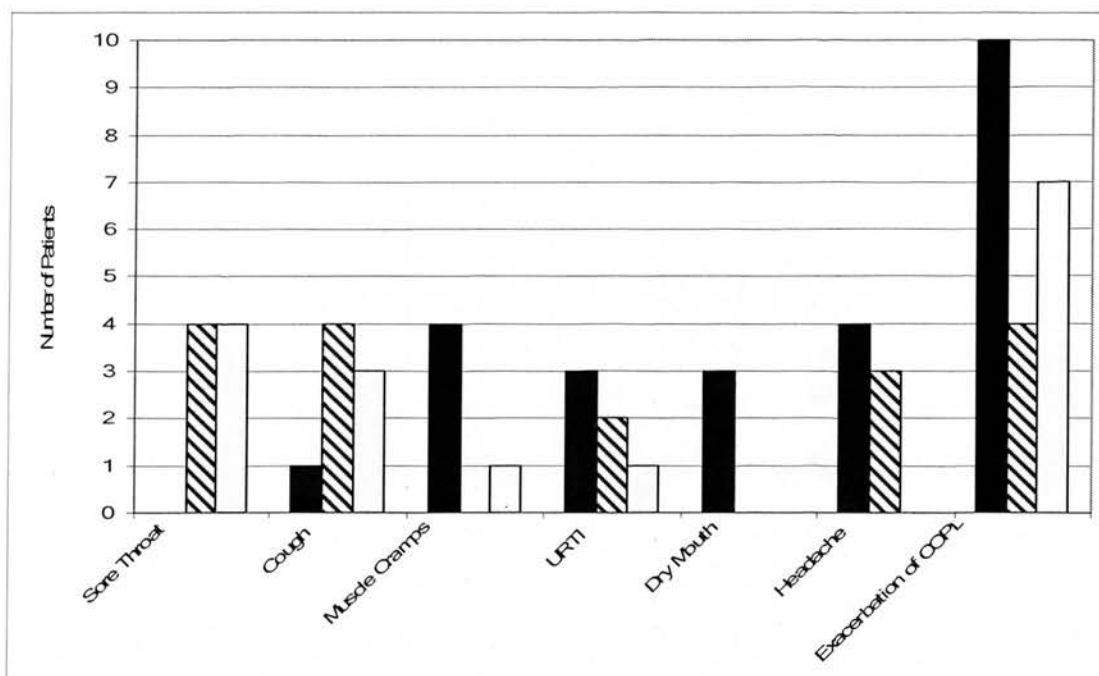


**Figure 5.1 Clinically significant exacerbations during trial**

Number of clinically significant exacerbations during trial per treatment group relative to the number of patients in each group (black histograms) expressed as the number of exacerbations (hatched histograms) and as the number of subjects who experienced an exacerbation (white histograms). \*  $p<0.05$  compared with placebo.

	Placebo n (%)	NAL 20mg bd n (%)	NAL 40mg bd n (%)
Randomised	19 (100)	20 (100)	19 (100)
Exacerbations	10 (53)	5 (25)	8 (42)

**Table 5.3 Exacerbations during trial per treatment group.**



**Figure 5.2 Number of Side Effects Noted During Trial.** Number of patients reporting the most commonly reported side effects experienced during the trial in each treatment group, 20mg bd (hatched histograms) and 40mg bd (white histograms), compared with placebo (black histograms). No statistically significant difference in reported side effects is noted between the three groups.

	Placebo n (%)	NAL 20mg bd n (%)	NAL 40mg bd n (%)
Randomised	19 (100)	20 (100)	19 (100)
Sore Throat	0 (0)	4 (20)	4 (21)
Cough	1 (5)	4 (20)	3 (16)
Headache	4 (21)	3 (15)	0 (0)
URTI	3 (16)	2 (10)	1 (5)
Muscle Cramp	4 (21)	0 (0)	1 (5)
Dry Mouth	3 (16)	0 (0)	0 (0)

**Table 5.4 Side Effects during trial per treatment group**

Number and percentage of patients who experienced the most frequently reported adverse events during the trial per treatment group

## 5.11 Spirometry

Spirometry was performed on all patients to assess suitability for trial entry. Post bronchodilator FEV<sub>1</sub> and FVC was assessed at baseline and after 4 and 12 weeks of trial therapy.

Results discussed below relate to those subjects who completed the trial. Results are expressed as absolute post bronchodilator FEV<sub>1</sub> in litres and also percentage predicted post bronchodilator FEV<sub>1</sub>. A wide range in FEV<sub>1</sub> was noted for each treatment arm prior to trial commencement with no significant difference between the three treatment arms.

Mean absolute FEV<sub>1</sub> in the placebo group dropped by 60ml from 1.41l to 1.35l during the duration of the trial, however this was not statistically significant ( $p>0.05$ ).

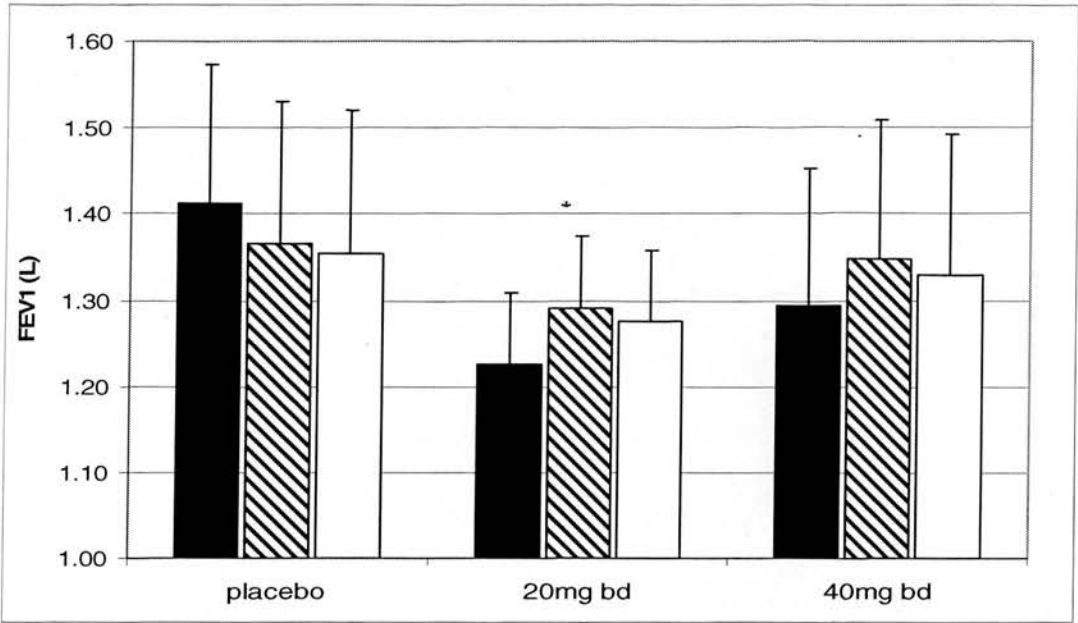
A small improvement in FEV<sub>1</sub> (60ml) was demonstrated in the 20mg bd NAL group at week 4 of the trial which was statistically significant when compared with baseline ( $p=0.022$ ). A trend towards improvement (40ml) was noted at the end of trial compared with baseline, but this did not achieve statistical significance ( $p=0.10$ ).

40mg bd NAL produced a small improvement in FEV<sub>1</sub>(l) at week 4 of trial therapy (60ml) which was statistically significant ( $p=0.021$ ) compared with baseline levels. There was no statistical difference between baseline levels and trial completion FEV<sub>1</sub> for this group.

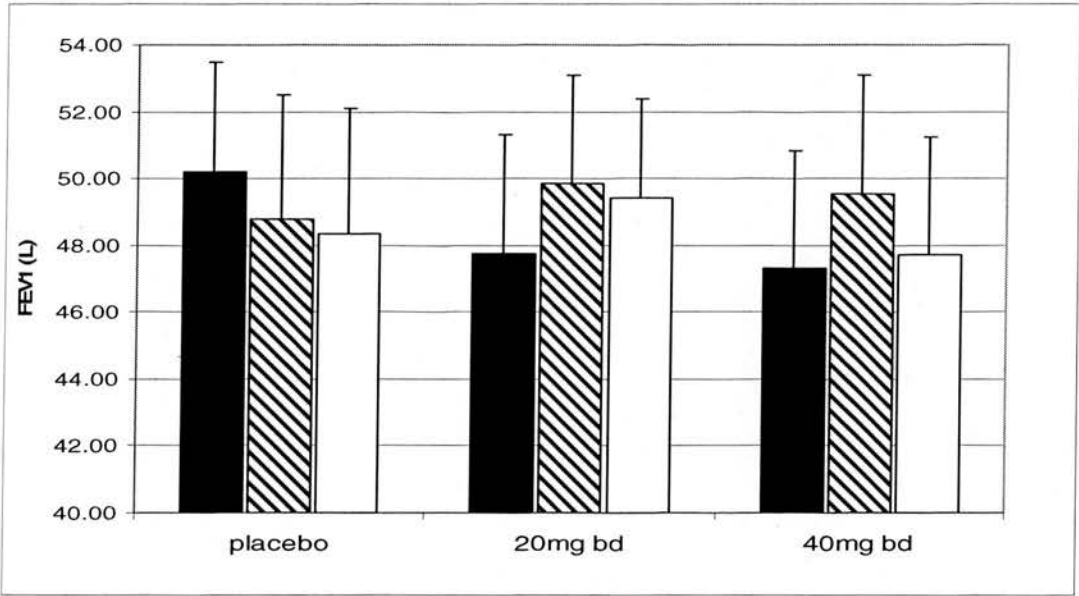
Analysis between treatment arms did not demonstrate any significant changes in FEV<sub>1</sub>.

In summary there was evidence of small changes in the FEV<sub>1</sub> within the treatment groups at week four of the study. However this was not demonstrated at trial completion. No significant impact on FEV<sub>1</sub> was shown when the various treatment arms were compared.





**Figure 5.3** Post Bronchodilator FEV<sub>1</sub> during trial in each treatment group. Histograms represent the mean and error bars the SEM. Black histograms represent week 0 of trial, hatched histograms week 4 and white histograms trial completion. \* =  $p < 0.05$  when compared with 20mg bd baseline. + =  $p < 0.05$  compared with 40mg bd baseline



**Figure 5.4** Post Bronchodilator FEV1 percentage predicted during trial in each treatment group. Expressed as mean for each group at each stage of the trial, error bars express standard error of mean. Black histograms represent week 0 of trial, hatched histograms week 4 and white histograms trial completion.

	Placebo n=15	20mg bd n=14	40mg bd n=13
Wk of trial	Post Bronchodilator FEV <sub>1</sub> (L) (MEAN ± SEM)		
0	1.41±0.16	1.23±0.08	1.29±0.16
4	1.37±0.16	1.29±0.08	1.35±0.16
12	1.35±0.17	1.28±0.08	1.33±0.16
	Placebo	20mg bd	40mg bd
	Post Bronchodilator Percentage Predicted FEV <sub>1</sub> (MEAN ± SEM)		
0	50.21±3.27	47.73±3.57	47.31±3.54
4	48.79±3.70	49.87±3.24	49.54±3.56
12	48.36±3.76	49.40±2.98	47.69±3.56

**Table 5.5** Post bronchodilator FEV<sub>1</sub> in litres and percentage predicted during course of trial for each treatment group. Values expressed as mean and standard error of mean

## 5.12 St Georges Respiratory Questionnaire

St Georges Respiratory Questionnaire (SGRQ) was completed at commencement of trial therapy (week 0), after 4 weeks of therapy and at trial completion. Patients who withdrew from the trial had an Early Termination visit (ET) at which a SGRQ was collected where possible.

SGRQ was available from all 58 participants at baseline. Ten patients withdrew prior to week 4, 8 of whom completed a SGRQ. 48 questionnaires were completed at week 4. Five subjects who withdrew between week 4 and week 12, 1 completed a SGRQ. SGRQ was obtained for 43 patients at trial completion. A breakdown of the SGRQ per treatment arm is listed below.

### Review of response to treatment

#### 5.12.1a Total Scores

Analysis of SGRQ changes in response to treatment was carried out only in those patients who provided SGRQ at Week 0, 4 and 12. Total scores and component scores were analysed. Analysis between the groups was carried out using ANOVA. Changes within treatment arms were analysed using Wilcoxon Matched Pairs analysis.

Prior to trial commencement, there were no significant differences between the treatment arms for any component of the SGRQ (table 5.6). Initial analysis of the changes was made between health status in the two treatment arms compared with placebo. Additional analysis of changes within the treatment arms was carried out.

SGRQ total score improved during the trial in the placebo group from 55.7( $\pm$ 13.5) at trial commencement to 51.6( $\pm$ 14.9) at week 4 and 50.3( $\pm$ 14.8) at trial completion, although this change was not statistically significant,  $p=0.09$ . Table 6.6, Figure 5.5d

The NAL 20mg bd produced a 6 point improvement in total score from 54.3 ( $\pm$ 18.8) to 48.3 ( $\pm$ 22.0) in total SGRQ score at week 4 of the trial compared with baseline. However this change was not statistically significant ( $p=0.08$ ). At trial completion the total score was clinically and statistically improved when compared with baseline measurements, from 54.3 ( $\pm$ 18.8) to 46.6 ( $\pm$ 18.2),  $p=0.02$ . Table 5.6, Figure 5.5d

NAL 40mg bd group produced an improvement in the total SGRQ from 51.45 ( $\pm$ 13.38) at baseline to 40.37 ( $\pm$ 13.63), at week 4,  $p=0.0012$ . At trial termination, week 12, the SGRQ total score was 49.05 ( $\pm$ 15.07) which was not statistically or clinically different from trial commencement, 51.45 ( $\pm$ 13.38). Table 5.6, Figure 5.5d.

The improvements in the SGRQ Total Score demonstrated in the 20mg bd group at week 12 and the 40 mg bd group at week 4 were statistically significant when compared with the baseline levels for these groups. However when scores were compared with placebo no statistically significant changes were noted. This may relate to the small sample size.

### 5.12.2 Component Scores

The SGRQ symptom component did not change during the trial in the placebo group. In contrast, an improvement of symptom score was seen at trial completion in the 20mg bd (-12.2 points,  $p=0.01$ ) and at week 4 in the 40mg bd (-13.8 points,  $p=0.001$ ) groups. At these two time points changes in symptom scores had the largest effect on total health status. A 12.3 point drop in symptom score in the 20mg bd group was noted at week 4 of the trial which was close to significance ( $p=0.09$ ). Table 5.6, Figure 5.5a, 5.6a.

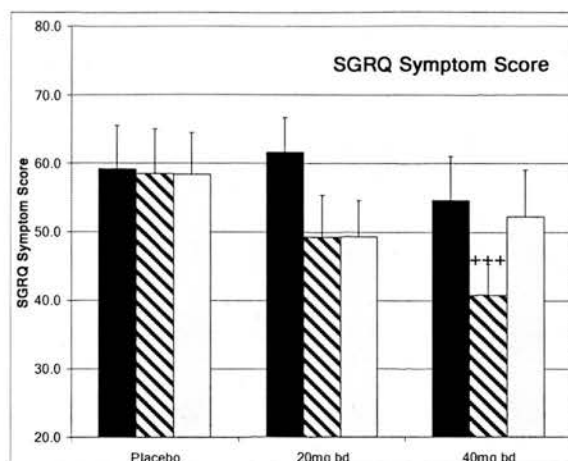
SGRQ Activity component changes were noted in the 40mg bd group at week 4 (-9.9 points,  $p=0.034$ ). No other statistically significant changes were noted at any time during the trial although the 5.4 point improvement in the placebo group at completion was close to statistical significance,  $p=0.07$ . Table 5.6, Figure 5.5a, 5.6a.

The impact component of the SGRQ improved by 10.8 points in the 40mg bd group at week 4 of the trial,  $p=0.008$ . An improvement was also seen in the placebo group at week 4, (-8.6 points,  $p=0.015$ ). At trial completion the improvement of 7.1 points in the impact score in the 20mg bd group was statically significant,  $p=0.049$ , whilst the improvement in the placebo group, also 7.1 points was close to significance ( $p=0.065$ ). In contrast the 40mg bd group impact score returned to near baseline levels.

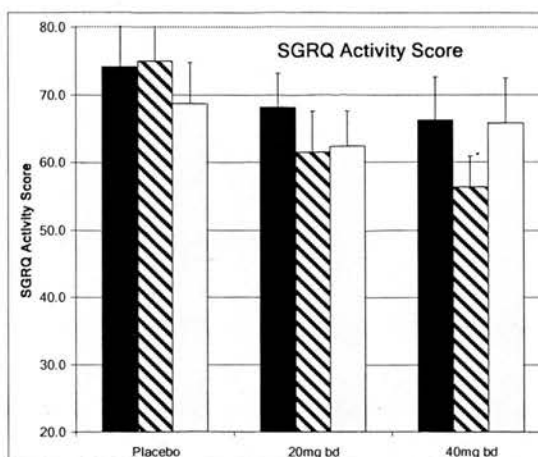
In summary the improvement in health status that was noted in the 40mg bd group at week 4 of the trial was related to all three components of the SGRQ. These changes were statistically significant when compared with baseline 40mg bd levels

but not when compared with placebo. In the 40mg bd treatment arm health status scores returned to baseline levels at trial completion.

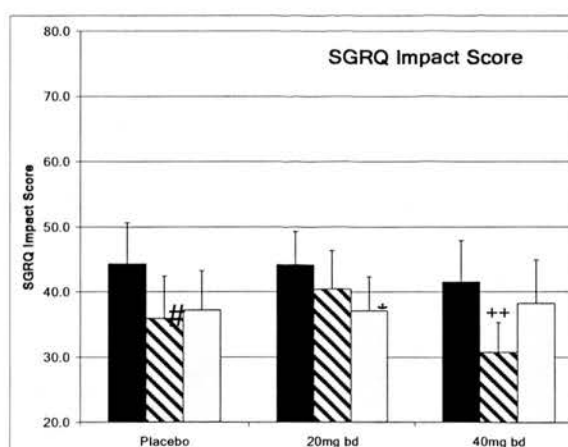
Treatment with 20mg bd NAL improved total score at trial completion with the symptoms component making the main contribution to this change. This was statistically and clinically significant when compared with the baseline levels in the 20mg bd group. However the changes in the 20mg bd group were not statistically significant when compared with the placebo group.



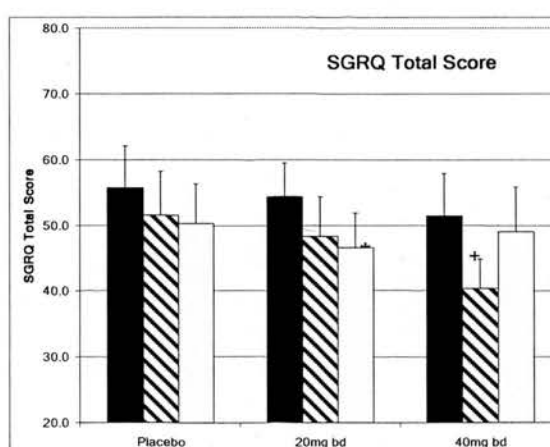
5.5a



5.5b



5.5c



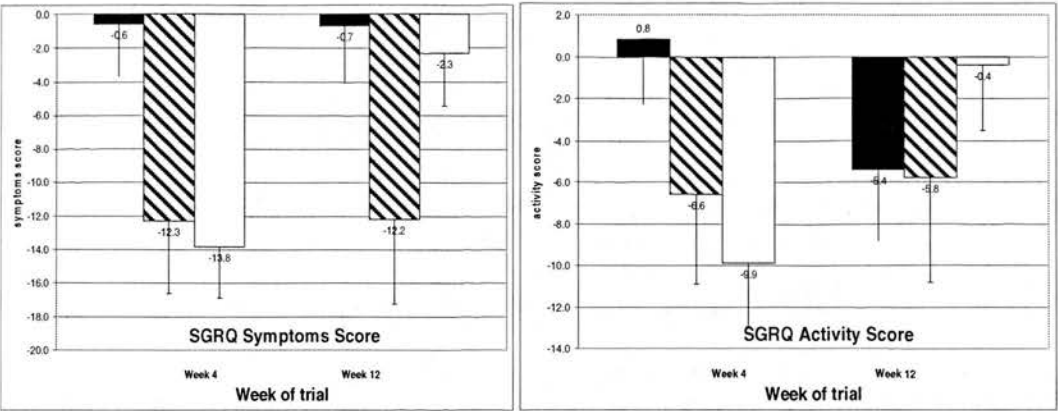
5.5d

**Figure 5.5** St Georges Respiratory Questionnaire scores during the trial for the three treatment groups. Scores are expressed as mean and SEM. Black histograms represent week 0 of trial, hatched histograms week 4 and white histograms trial completion. The three components of the SGRQ; symptoms (a), activity (b) and impact (c) of disease along with total scores (d) during the trial for the three treatment groups. \*= $p < 0.05$  vs 20mg bd baseline, += $p < 0.05$  vs 40mg bd baseline, ++= $p < 0.01$  vs 40mg bd baseline, +++= $p < 0.001$  vs 40mg bd baseline, #= $p < 0.05$  vs baseline placebo.

SGRQ Component	Week of Trial	Placebo <sub>++</sub> mean, sem	p vs baseline placebo	20mg bd mean, sem	p vs baseline 20mg bd	40mg bd mean, sem	p vs baseline 40mg bd
Symptoms	0	59.1±6.4		61.6±5.0		54.61±6.4	
	4	58.5±6.5	0.900	49.3±6.0	0.090	40.80±4.5	<b>0.001</b>
	12	58.4±6.0	0.700	49.4±5.2	<b>0.010</b>	52.28±6.7	0.400
Activity	0	74.2±3.7		68.2±4.6		66.22±5.5	
	4	75.0±3.7	0.900	61.6±7.2	0.150	56.33±6.3	<b>0.034</b>
	12	68.8±4.3	0.070	62.4±6.8	0.430	65.82±6.1	0.900
Impact	0	44.3±4.6		44.2±5.7		41.50±3.7	
	4	35.9±4.1	<b>0.015</b>	40.3±5.8	0.190	30.75±4.1	<b>0.008</b>
	12	37.1±3.9	0.065	37.1±5.3	<b>0.049</b>	38.19±4.9	0.274
Total	0	55.7±3.5		54.3±4.9		51.45±3.6	
	4	51.6±3.8	0.060	48.3±5.7	0.080	40.37±3.6	<b>0.012</b>
	12	50.3±3.8	0.090	46.6±4.7	<b>0.022</b>	49.05±4.0	0.330

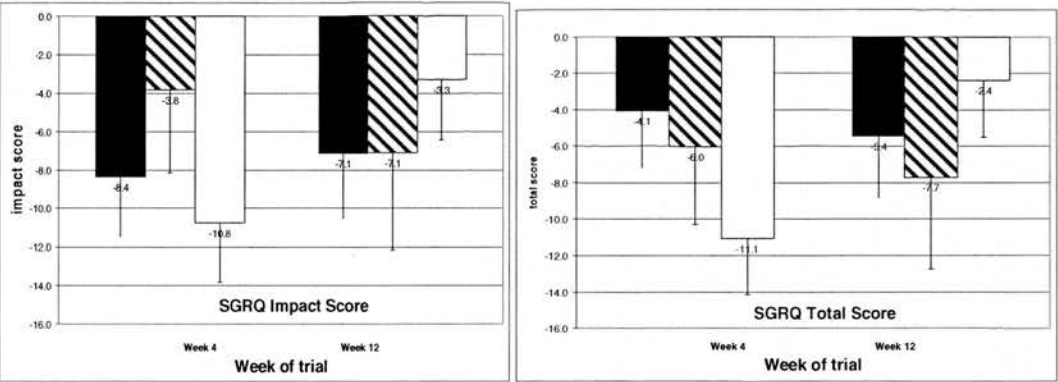
Table 5.6 St Georges Respiratory Questionnaire score during trial for each treatment group. Figures are expressed as mean of group and standard error of mean. Total SGRQ score is expressed along with symptom, activity and impact component of score. The p value is expressed for each stage of the trial compared with the baseline for that group. Significant p values are in bold.





5.6a

5.6b



5.6c

5.6d

**Figure 5.6 SGRQ Symptom (a), Activity (b), Impact (c) and Total Scores (d).** Change from Baseline at Weeks 4 and 12 of trial for the three treatment groups. Mean SGRQ score was calculated for each treatment group at each of the three study visits; baseline, after 4 weeks and 12 weeks of therapy. The baseline score was then subtracted from the mean score at weeks 4 and 12 of the trial for each treatment group. The three components of the SGRQ are expressed along with the total score. Histograms represent the mean changes from baseline for placebo (black), 20mg bd NAL (hatched) and 40mg bd (white).

## 5.13 Symptom Diary Cards

### 5.13.1 Symptom Diary Cards Overview

All subjects who participated in the trial completed a daily diary of symptoms throughout the trial. Full sets of diary card responses were obtained for thirteen subjects in the 40mg NAL bd group, 15 of the 20mg NAL bd subjects and 15 of the placebo subjects.

Trial participants were asked to record scores for cough (0=no cough to 3=cough throughout day), breathlessness (0=no breathlessness to 4=breathlessness at rest), sputum production (0=no sputum to 3=sputum throughout day), sputum colour (0=no sputum to 4 dark green/ yellow sputum), number of night awakenings, time off work, time unable to carry out household tasks and any changes required in medications. They were also encouraged to record any new or changing symptoms which they experienced during the course of the trial. An example of the daily diary card is found in appendix 5.4.

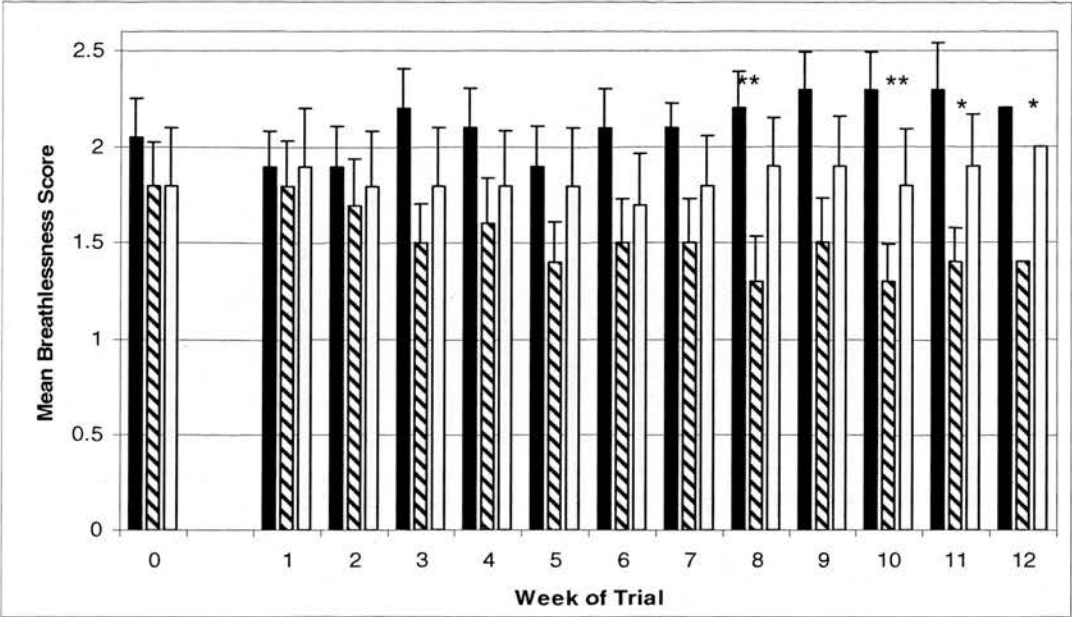
The mean of symptom scores for each week of the trial were calculated for each individual. The average score for each treatment arm per week of the trial was then calculated. Comparisons were then made between baseline and trial completion within treatment groups and between placebo and active treatment for the duration of the trial.

Baseline scores were taken as the average score for the two weeks prior to trial commencement. Diary card scores were recorded for breathlessness, cough, sputum production and sputum colour. Further analysis was performed to calculate of the number of days per week with no or mild symptoms for breathlessness, cough, sputum production, sputum colour and night time awakenings.

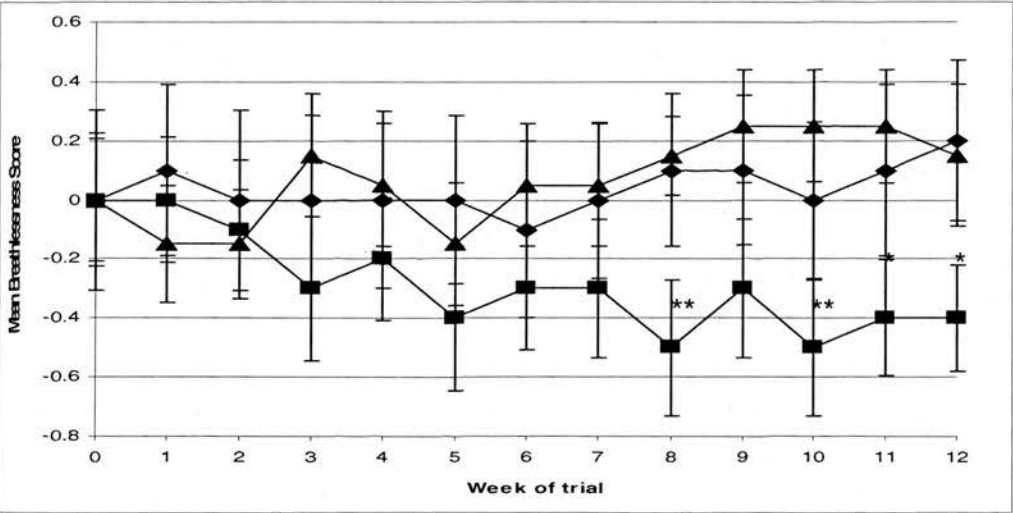
### 5.13.2 Diary Card Breathlessness Score

Subjects were asked to record daily breathlessness score from 0, no breathlessness to 4, breathless at rest (appendix 5.4). Mean breathlessness score per treatment arm per week prior to trial commencement was 1.8 ( $\pm 1.3$ ) for 40mg bd NAL, 1.8 ( $\pm 1.0$ ) for 20mg bd NAL and 2.0 ( $\pm 0.9$ ) for Placebo with no significant difference between the three groups.

Analysis within treatment arms did not show any statistically significant change in breathlessness scores from trial commencement to completion for the 40mg bd NAL or the placebo group. A statistically significant improvement in breathlessness score was noted in the NAL 20mg bd group of between 0.3 and 0.5 at weeks 8 ( $p=0.004$ ), 10 ( $p=0.004$ ), 11 ( $p=0.04$ ) and 12 ( $p=0.04$ ) of the trial. The mean score at last week of trial was 1.4 (0.8) an improvement of 0.4 from baseline, Figures 5.7, 5.8.



**Figure 5.7 Mean Breathlessness score for each treatment group during the trial.** Histograms represent the mean weekly breathlessness scores for all individuals who completed the trial in each of the treatment groups during the trial, placebo (black histograms), 20mg bd (hatched histograms) and 40mg bd (white histograms) Error bars express the standard error of mean. Significant improvement in the 20mg bd group compared with baseline levels was noted at weeks 8, 10, 11 and 12 of the trial. No significant change was noted in the 40mg bd group or the placebo group. \*  $p < 0.05$  compared with 20mg bd NAL baseline levels. \*\* $p < 0.005$  compared with 20mg bd NAL baseline



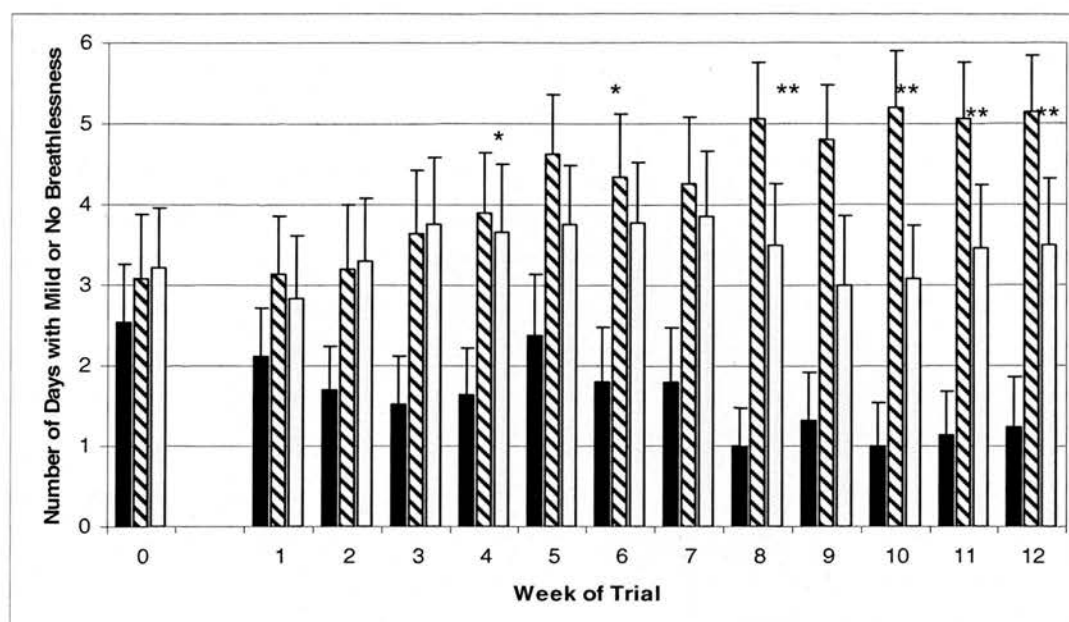
**Figure 5.8 Change from baseline levels mean breathlessness score for the three treatment arms, 40mg bd NAL (diamond), 20mg bd NAL (square) and placebo (triangle).** Mean Breathlessness Score at week 0 is subtracted from the breathlessness score for each week of the trial, negative figures indicate improvements in mean score during trial. Error bars represent standard error of mean. Breathless score significantly improved from baseline at weeks 8, 10, 11 and 12 of the trial in the 20mg bd group. \*  $p < 0.05$  compared with 20mg bd NAL baseline levels. \*\* $p < 0.005$  compared with 20mg bd NAL baseline levels.

### 5.13.3 Number of Days with Mild or No Breathlessness

The impact of treatment on subject reporting of breathlessness was also expressed as the mean number of days per week subjects in each treatment arm experienced mild or no breathlessness, equivalent to diary card score 0 or 1.

The 40mg bd and placebo groups did not show any change in the average number of days per week with mild or no breathlessness throughout the study period. The mean number of days per week with mild or no breathlessness in the 20mg bd NAL treatment arm steadily increased during the trial from 2.9 ( $\pm 3.3$ ) days per week prior to trial commencement to 5.1 ( $\pm 3.0$ ) days per week at trial completion. A statistically significant difference between 20mg bd and placebo was noted at weeks 4, 6 and 8-12 of the trial. Figures 5.9, 5.10

These data suggest that treatment with low dose NAL (20mg bd) significantly improves the number of days per week with no or mild symptoms of breathlessness compared with placebo.



**Figure 5.9 Mean Number of Days per Week with Mild or No Breathlessness per Treatment Arm.** Histograms represent the mean number of days per week with diary card reporting of mild or no breathlessness in the three treatment groups during the trial, placebo (black histogram), 20mg bd (hatched histogram) and 40mg bd (white histogram) for each week of the trial. Error bars represent the standard error of mean. Significant improvement in the 20mg bd compared with placebo was noted at weeks 4, 6, 8, 10-12 of the trial. \*  $p < 0.05$  vs placebo for week 4 and 6 of the 20mg bd group and \*\* $p < 0.005$  vs placebo for weeks 8-12 of the trial group.

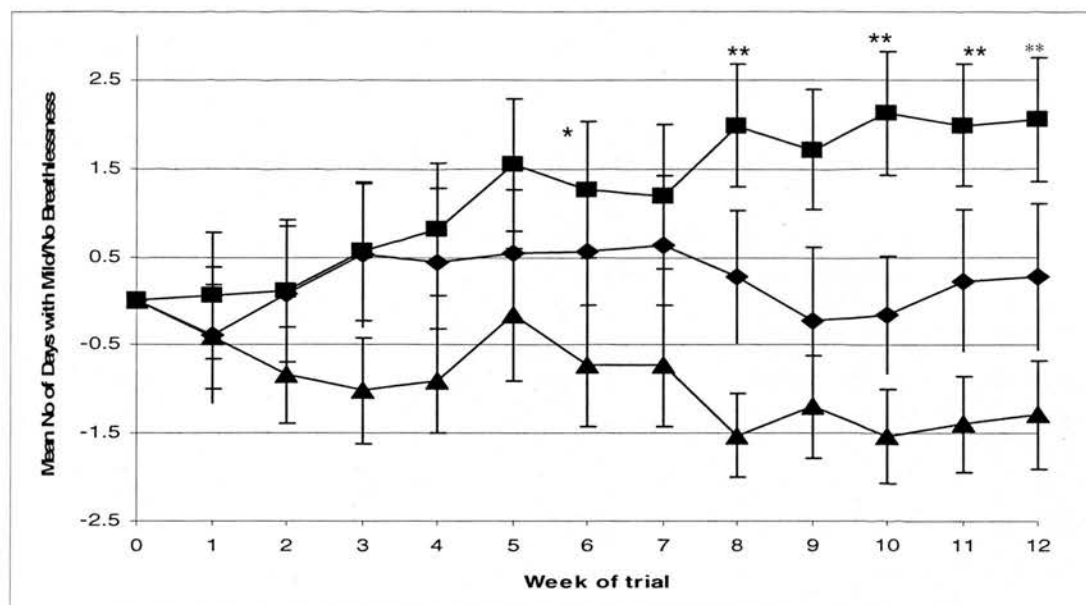


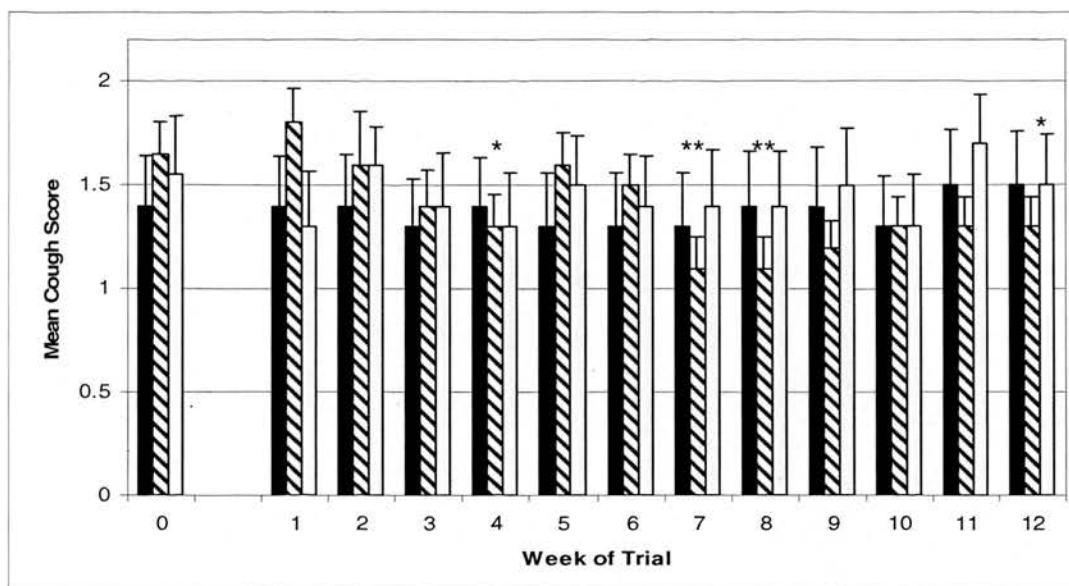
Figure 5.10 Change from baseline in the mean number of days with mild or no breathlessness in the treatment groups. Mean number of days with mild or no breathlessness for each week of the trial are subtracted from baseline levels, negative value indicates increasing number of days with breathlessness. Error bars represent the standard error of mean. Number of days per week with mild or no breathlessness significantly improved compared with placebo at weeks 4, 6, 8, 10-12 of the trial.  $*=p<0.05$ ,  $**=p<0.005$



#### 5.13.4 Cough Score

Diary card responses for cough score ranged from 0, no cough to 3, cough throughout day, appendix 6.4. Treatment with NAL 40mg bd or placebo did not affect the reporting of cough in the patient diary cards. NAL 20mg bd demonstrated a statistically significant improvement of between 0.35 and 0.45 from baseline at weeks 4, 7, 8 and 12 of the trial, Figure 5.11, 5.12.

These changes noted in the 20mg bd group were not statistically significant when compared with placebo.



**Figure 5.11 Mean Cough Score during trial per treatment group.** Average cough scores were calculated for each treatment group at each week of the trial. Histograms represent the mean weekly cough scores for all individuals who completed the trial in each of the treatment groups during the trial, placebo (black histograms), 20mg bd (hatched histograms) and 40mg bd (white histograms). Error bars represent the standard error of mean. Significant improvement in the 20mg bd group compared with baseline levels was noted at weeks 4, 7, 8 and 12 of the trial. No significant change was noted in the 40mg bd group or the placebo group. \*  $p < 0.05$  compared with 20mg bd NAL baseline levels. \*\* $p < 0.005$  compared with 20mg bd NAL baseline levels.

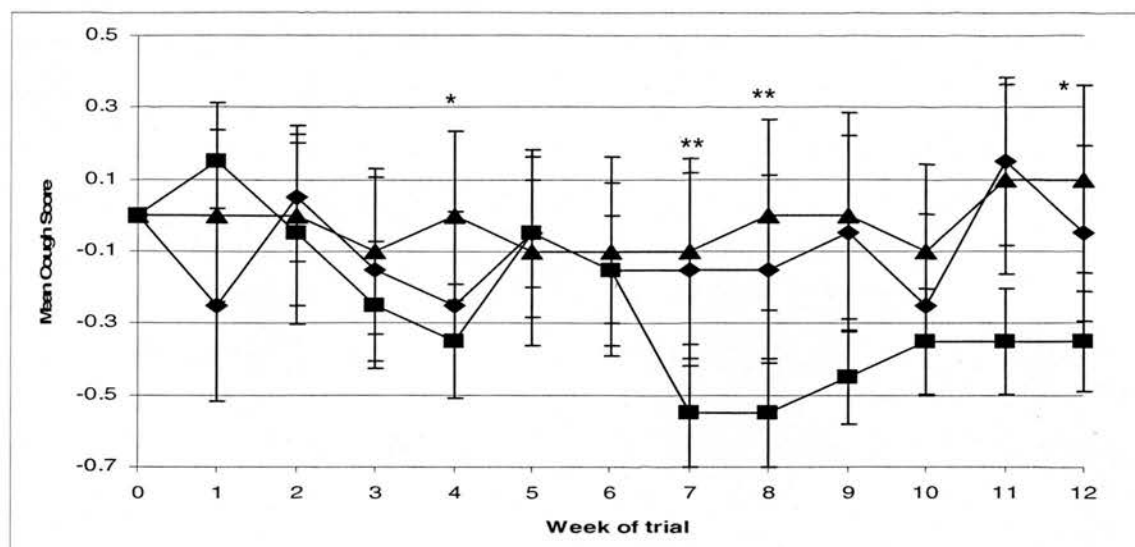


Figure 5.12 Change from baseline levels mean cough score for the three treatment arms, 40mg bd (diamond), 20mg bd (square) and placebo (triangle). Mean Cough Score at trial commencement was subtracted from the cough score for each week of the trial, negative figures indicate improvements in mean score during trial. Error bars represent standard error of mean. Cough score significantly improved from baseline at weeks 4, 7, 8 and 12 of the trial in the 20mg bd group. \*  $p < 0.05$  compared with 20mg bd NAL baseline levels. \*\*  $p < 0.005$  compared with 20mg bd NAL baseline levels.

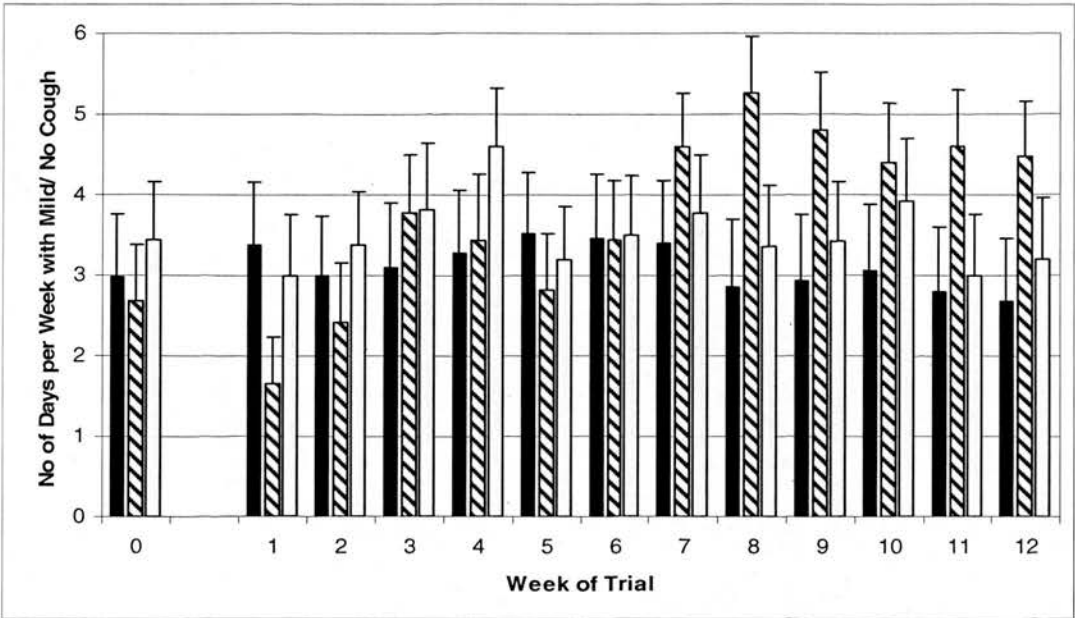
### 5.13.5 Number of days per week with mild/ no cough

The effect of treatment on patient diary card recording of cough was also expressed as the number of days per week with no or mild cough equivalent to cough score 0 or 1.

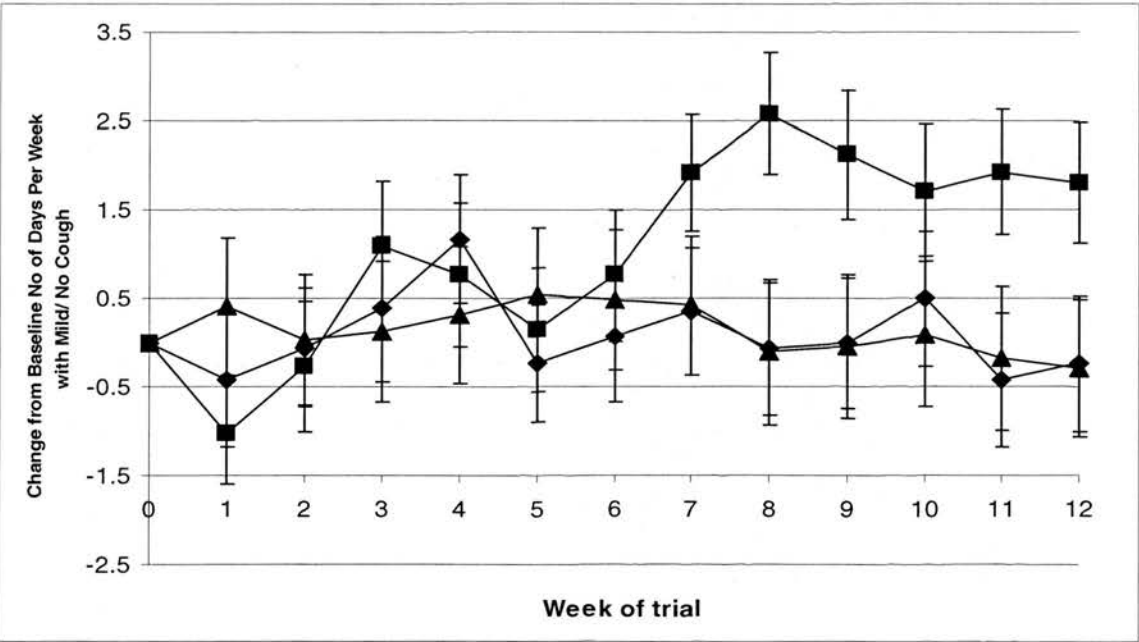
Average weekly cough score did not change significantly in any group during the trial. A comparison of changes with the active treatment and the placebo was also not significant

Analysis of the 20mg bd group data separately did demonstrate that the change from baseline was close to statistical significance at week 8 of the trial ( $p=0.057$ ).

Figure 5.13,5.14



**Figure 5.13 Number of Days per Week with Mild or No Cough per Treatment Arm.** Histograms represent the mean number of days per week with diary card reporting of mild or no cough in the three treatment groups, placebo (black histogram), 20mg bd (hatched histogram) and 40mg bd (white histogram) for each week of the trial. Error bars express the standard error of mean. No significant improvement is seen between the treatment groups during the trial.



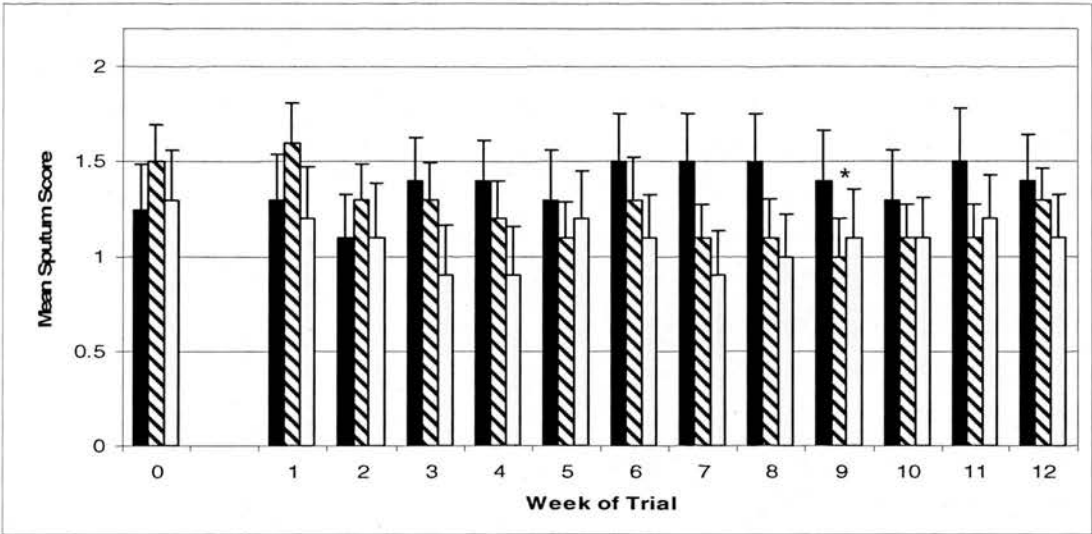
**Figure 5.14 Change from baseline levels mean number of days with no or mild cough for the three treatment arms, 40mg bd (diamond), 20mg bd (square) and placebo (triangle).** Mean number of days with no or mild cough at trial commencement was subtracted from the number of days with no or mild cough for each week of the trial, positive figures indicate improvements in mean score during trial. Error bars represent standard error of mean.

#### 5.13.6 Sputum Production

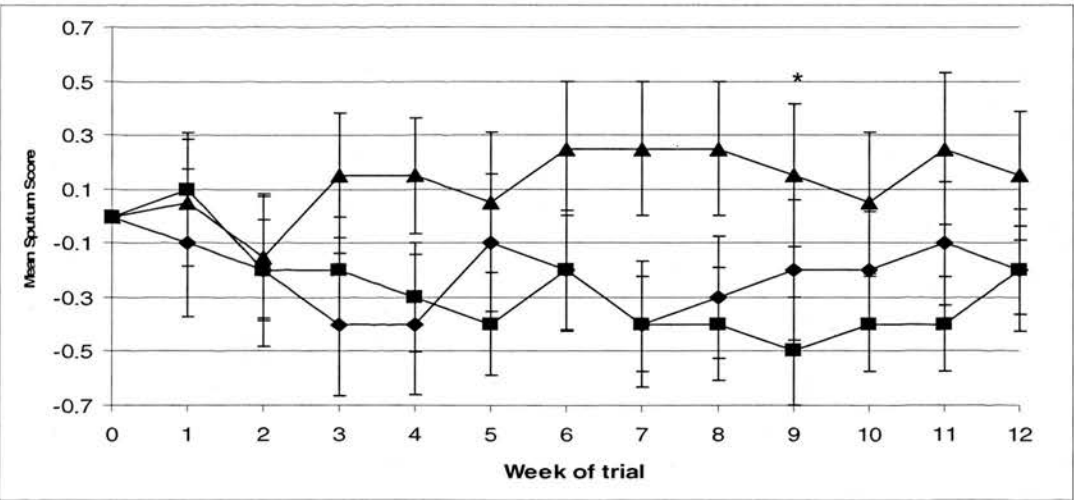
Subjects were asked to score daily sputum production, ranging from 0, no sputum, to 3, sputum throughout the day. There was a wide variation between individuals in the levels of sputum production before the start of the study. Despite this, and the small numbers in the trial, trends towards improvement in sputum production were seen for both treatment groups.

There was no significant change in the sputum production scores in the placebo arm of the study. In the 20mg bd group there was a trend towards a fall in sputum production during the trial. This was statistically lower than the 20mg bd baseline levels at week 9 of the trial,  $p=0.027$ . The 40mg bd NAL group demonstrated a non significant trend towards improved sputum production score at weeks 3, 4 and 7.

There were no significant differences in sputum production comparing placebo group and active treatment groups. Figure 5.15, 5.16.



**Figure 5.15 Mean Sputum Production score for each treatment group during the trial.** Histograms represent the mean weekly sputum production scores for all individuals who completed the trial in each of the treatment groups during the trial, placebo (black histograms), 20mg bd (htched histograms) and 40mg bd (white histograms). Error bars represent the standard error of mean. Significant improvement in the 20mg bd group compared with baseline levels was noted at week 9 the trial. No significant change was noted in the 40mg bd group or the placebo group. \*  $p < 0.05$  compared with baseline levels.



**Figure 5.16 Change from baseline levels mean sputum production score for the three treatment arms, 40mg bd (diamond), 20mg bd (square) and placebo (triangle).** Mean score at trial commencement was subtracted from the score for each week of the trial, negative figures indicate improvements in mean score during trial. Error bars represent the standard error of mean. Sputum production score significantly improved from baseline at week 9 of the trial in the 20mg bd group. \*  $p < 0.05$  compared with baseline levels.

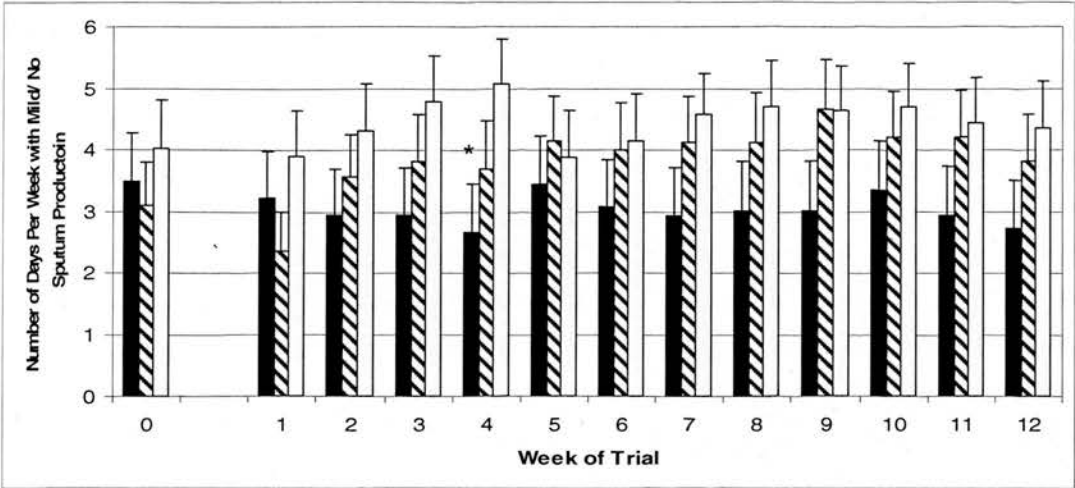
### 5.13.7 Number of days with mild or no sputum production

Sputum production is also expressed as the number of days per week with mild or no sputum production, equivalent to sputum scores 0 or 1. These results run in parallel with the sputum production scores.

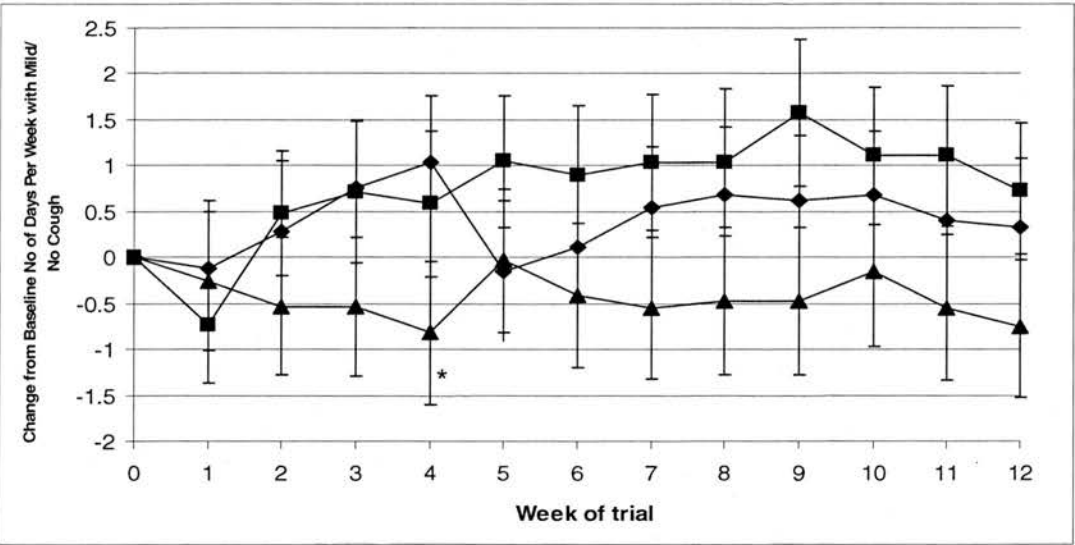
A trend towards improvement from baseline was seen in the 40mg bd and 20mg bd groups. Again this improvement was most marked early in the trial with the 40mg bd treatment arm, a 1.0 improvement at week 4 in the number of days with mild or no sputum production, and later in the 20mg bd group with a 1.6 improvement in the number of days with mild or no sputum production at week nine. The average number of days with mild or no sputum production tended to fall from baseline in the placebo group.

At week 4 of the trial the increase in the number of days with mild or no sputum for those subjects taking 40mg bd NAL was statistically significant ( $p < 0.05$ ) compared with placebo. No other statistically significant differences were found. Figure 5.17, 5.18.





**Figure 5.17** Mean Number of Days per Week with Mild or No Sputum Production in each treatment group during the trial. Histograms represent the mean scores for all individuals who completed the trial in each of the treatment groups during the trial, placebo (black histograms), 20mg bd (hatched histograms) and 40mg bd (white histograms). Error bars represent the standard error of mean. Significant improvement in the 40mg bd group compared with placebo was noted at week 4 of the trial. No significant change was noted in the 20mg bd group or the placebo group. \*  $p < 0.05$  compared with baseline levels.

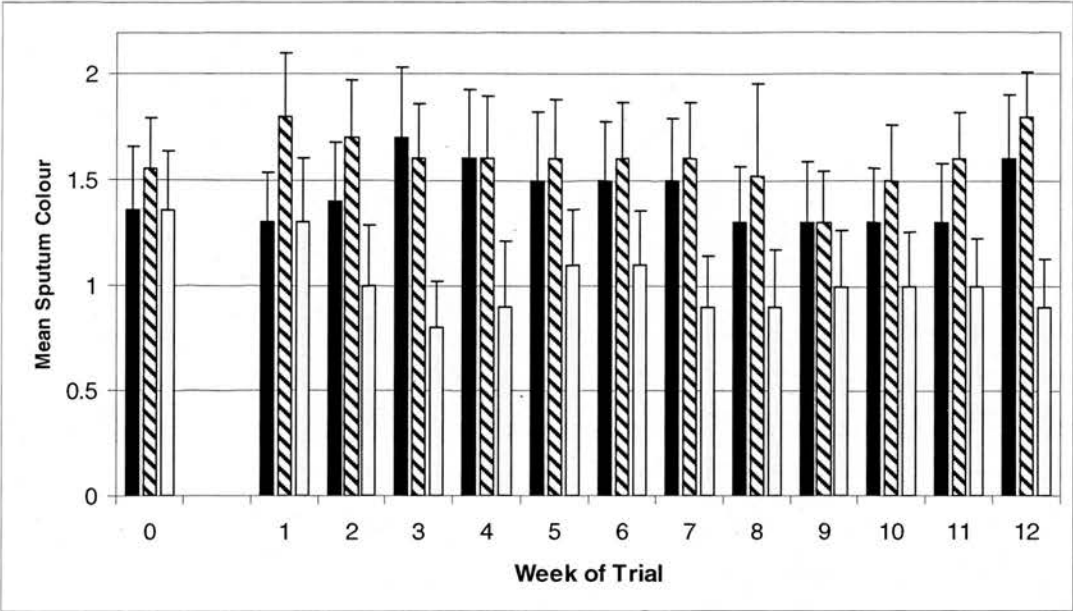


**Figure 5.18** Change from baseline levels mean number of days with mild or no sputum production for the three treatment arms, 40mg bd (diamond), 20mg bd (square) and placebo (triangle). Mean Score at trial commencement was subtracted from the score for each week of the trial, positive figures indicate improvements in mean score during trial. Error bars represent the standard error of mean. The number of days with mild or no sputum production significantly improved from baseline at week 4 of the trial in the 40mg bd group. \*  $p < 0.05$  compared with baseline levels.

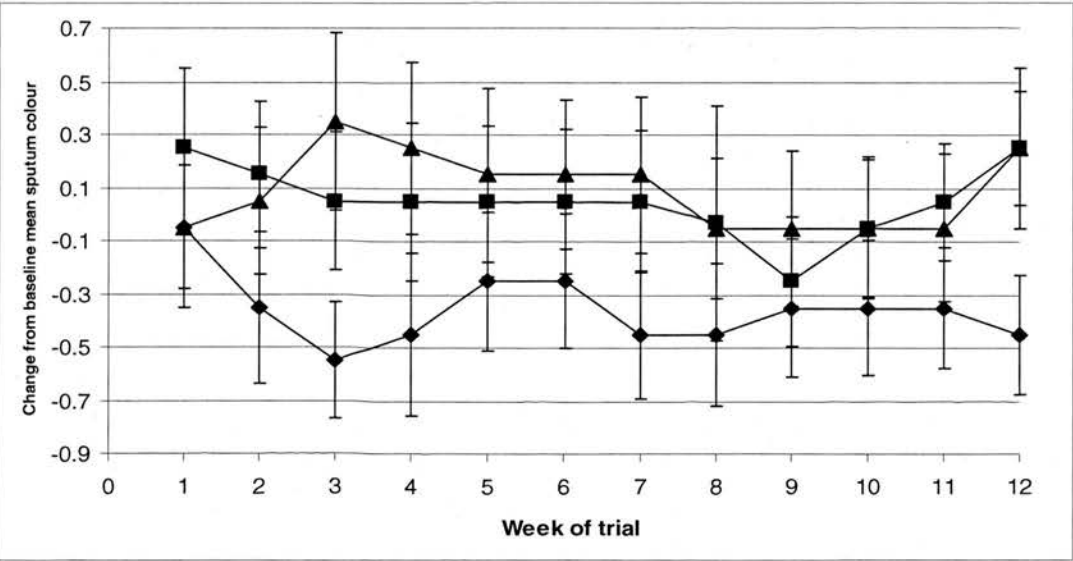
### 5.13.8 Daily sputum colour

The 40mg bd treatment group demonstrated a trend towards improvement in sputum colour scoring throughout the trial. This was most marked in the early stages of the trial, and was close to statistical significance compared with placebo at weeks 2 and 4 of the trial. Compared with other parameters measured in the 40mg group, the change demonstrated in sputum colour score did not return to baseline as the trial progressed.

In contrast a similar trend towards improvement in sputum colour was not shown in the 20mg bd NAL treatment group. Figure 5.19, 5.20



**Figure 5.19 Mean Sputum Colour score for each treatment group during the trial.** Histograms represent the mean weekly scores for all individuals who completed the trial in each of the treatment groups during the trial, placebo (black histograms), 20mg bd (hatched histograms) and 40mg bd (white histograms). Error bars represent the standard error of mean. No significant improvement was noted during the trial.

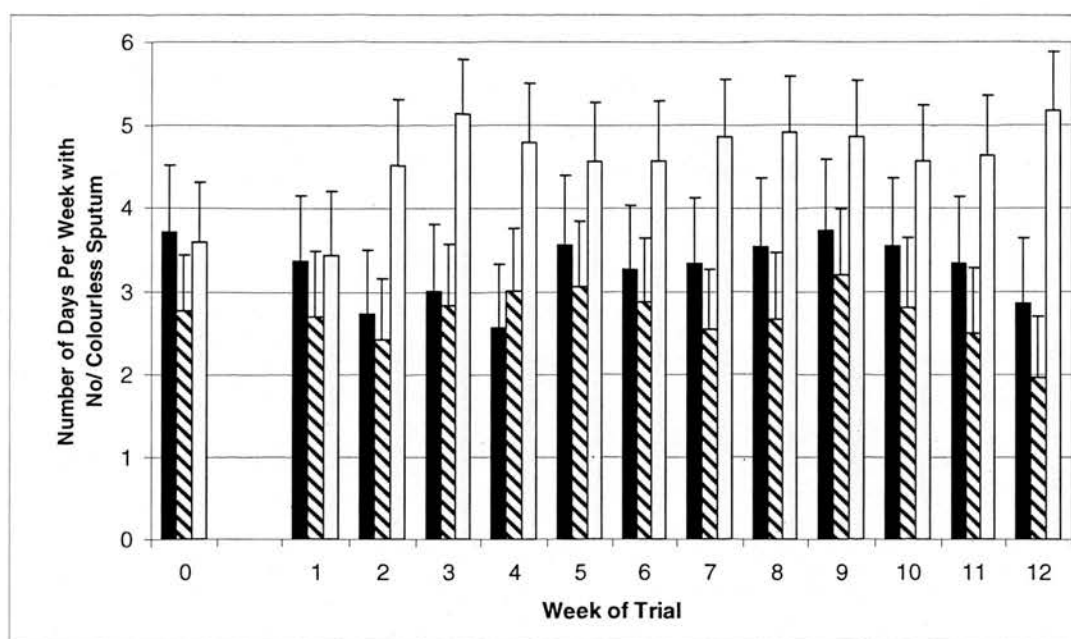


**Figure 5.20 Change from baseline levels mean sputum colour score for the three treatment arms, 40mg bd (diamond), 20mg bd (square) and placebo (triangle).** Mean Score at trial commencement was subtracted from the score for each week of the trial, negative figures indicate improvements in mean score during trial. Error bars represent the standard error of mean. No significant changes were noted during the trial.

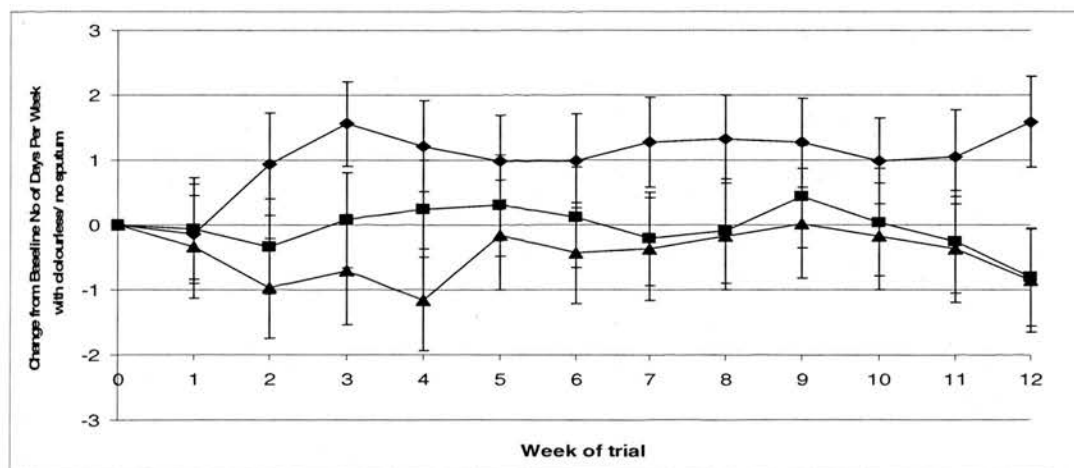
#### 5.13.9 Average number of days per week with colourless/ no sputum

These data are similar to those for the sputum colour scores. A trend towards improvement in the number of days with no or colourless sputum is seen in the 40mg treatment arm, which is not statistically significant ( $p=0.058$  at week 4) compared with placebo. No similar trend was seen in the 20mg bd group. Figure

5.21, 5.22



**Figure 5.21 Mean Number of Days per Week with Colourless or no sputum for each treatment group during the trial.** Histograms represent the mean weekly scores for all individuals who completed the trial in each of the treatment groups during the trial, placebo (black histograms), 20mg bd (hatched histograms) and 40mg bd (white histograms). Error bars represent the standard error of mean. No significant improvement was noted during the trial



**Figure 5.22 Change from Baseline levels mean Number of Days per Week with Colourless or No Sputum for the three treatment arms, 40mg bd (diamond), 20mg bd (square) and placebo (triangle).** Mean Score at trial commencement was subtracted from the score for each week of the trial, positive figures indicate improvements in mean score during trial. Error bars represent the standard error of mean. No significant changes were noted during the trial.

#### 5.13.10 Diary Card Summary

In summary, analysis of diary card scores indicated that the 20mg NAL bd treatment arm showed a significant improvement in the reporting of breathlessness when compared with placebo. A similar change was shown early in the trial for 40mg bd NAL but this was not sustained throughout the trial. Trends towards improvement in cough were seen. No improvement was demonstrated in terms of sputum production or colour nor any improvement in activities.

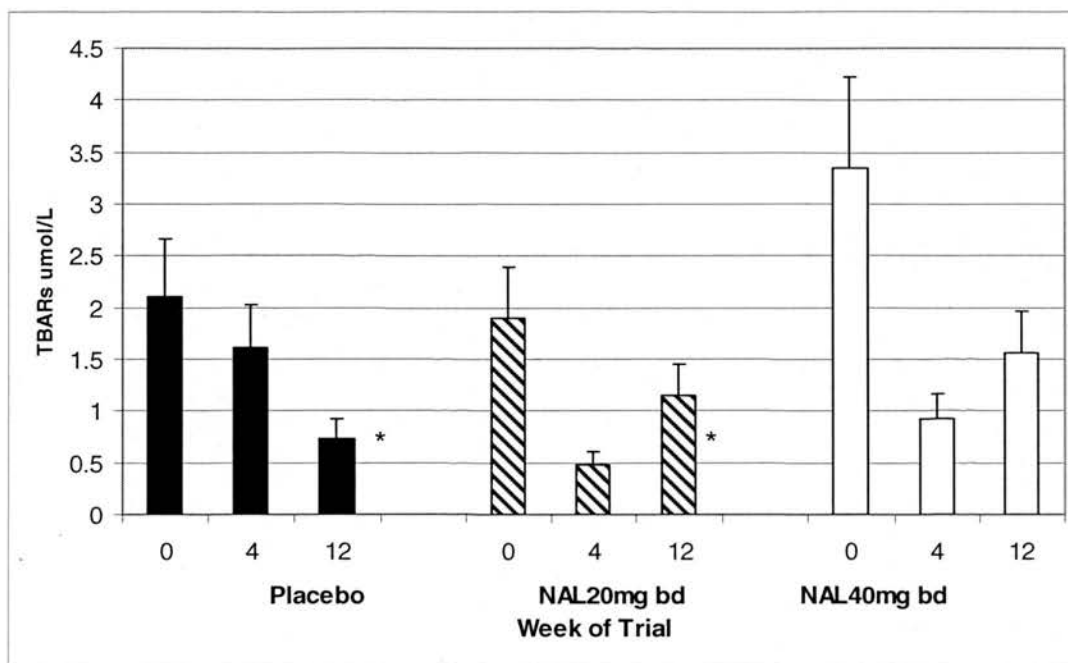
## **5.14 Plasma**

Plasma samples were obtained at trial entry (week -4 or -2), week 4 and week 12, trial completion.

### **5.14.1 Thiobarbituric Acid Reactive Substances**

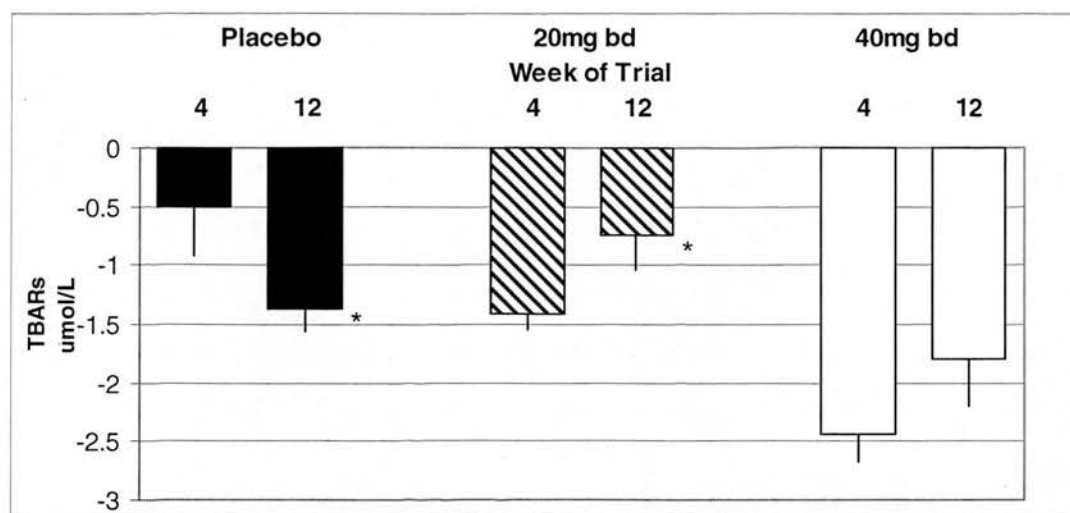
A wide range of Thiobarbituric Acid Reactive Substance (TBARS) levels in plasma was noted in each of the treatment arms prior to trial commencement, with no significant differences in the levels between the three treatment groups. This between-subject variation was noted for the duration of the trial.

A statistically significant ( $p=0.049$ ) drop in the levels of TBARS measured in the placebo group was noted from baseline to trial completion,  $2.11\mu\text{mol/l} \pm 3.2$  versus  $0.74\mu\text{mol/l} \pm 1.38$ . Average plasma levels of TBARS in the 20mg bd group decreased from a baseline of  $1.9\mu\text{mol/l} \pm 3.49$  to  $1.15\mu\text{mol/l} \pm 2.8$  at week 12. There was no significant change in TBARS levels in the 40mg bd group. When between treatment arm analysis was performed there was no statistically significant difference between the three groups.



**Figure 5.23 Plasma TBARS levels in the three treatment groups.** Plasma was obtained at baseline and weeks 4 and 12 of the trial. Histograms represent the mean in three treatment groups, placebo (black bars), NAL 20mg bd (hatched bars) and NAL 40mg bd (white bars). Error bars represent the standard error of mean. A significant drop was demonstrated in plasma TBARS in the Placebo and 20mg bd groups between baseline and week 12. \* $p < 0.05$

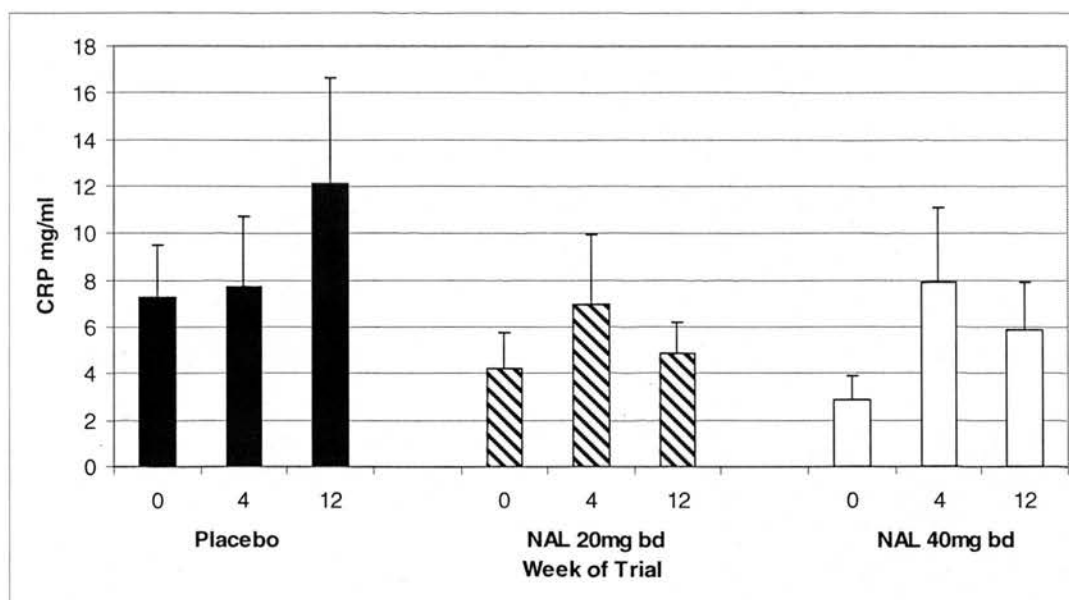




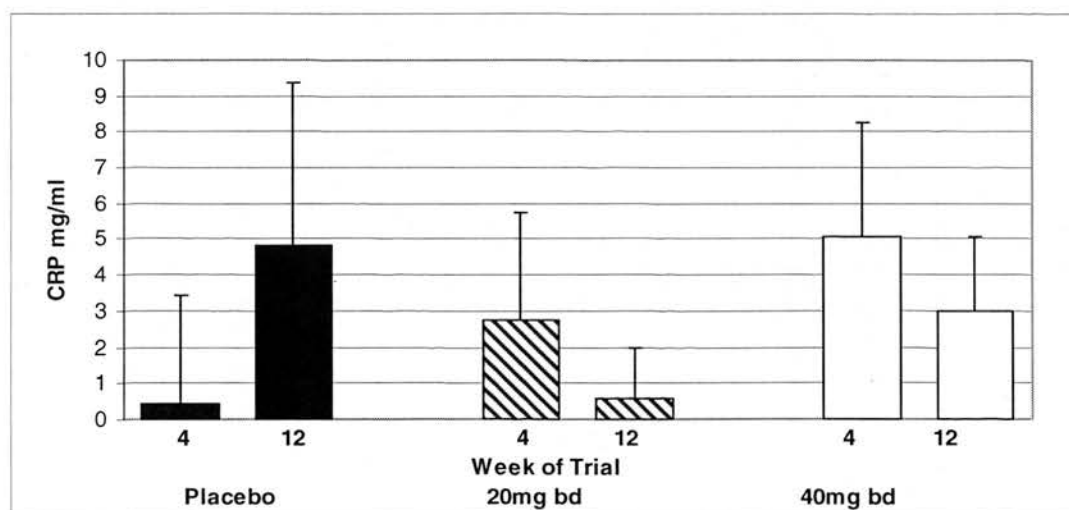
**Figure 5.24 Change from baseline mean plasma TBARS.** Histograms represent the means measured at weeks 4 and 12 of the trial after subtraction of the baseline levels. Negative values represent a decrease in the level of TBARS measured during the trial. Black histograms represent placebo, hatched 20mg bd and white 40mg bd. Error bars express standard error of mean. A statistically significant fall in the plasma TBARS was shown at week 12 of the trial compared with baseline in the placebo and 20mg bd groups.  $*=p<0.05$

#### **5.14.2 C-Reactive Protein (CRP)**

A wide variation in the level of CRP measured in plasma was noted within each of the treatment arms at the start of trial. No significant variation was noted between treatment arms at trial commencement and no significant change was noted in levels of CRP between treatment arms during the course of the trial. Inhaled NAL had no effect on plasma CRP during the trial. Furthermore prior use of ICS did not affect plasma levels of CRP.



**Figure 5.25 Plasma CRP levels in plasma during trial.** Plasma was obtained at baseline and weeks 4 and 12 of the trial. Histograms represent the mean CRP (mg/ml) measured in plasma in the three treatment groups, placebo (black bars), NAL 20mg bd (hatched bars) and NAL 40mg bd (white bars) during the trial. Error bars represent the standard error of mean. No significant change was demonstrated in plasma CRP in the three treatment groups.



**Figure 5.26 Change from baseline plasma CRP during trial.**

Histograms represent the mean CRP measured at weeks 4 and 12 of the trial after subtraction of the baseline mean, positive values represent an increase in the level of CRP measured compared with baseline. Black histograms represent placebo, hatched 20mg bd and white 40mg bd. Error bars represent standard error of mean.

5.15 Induced Sputum

Absolute and differential cell counts were obtained from induced sputum and sputum supernatant was analysed for the cytokines IL-6, IL-8, TNF- $\alpha$ , IL- $\beta$  and VEGF.

5.15 Sputum Production

47 of the 58 (81%) patients recruited into the trial were able to provide sputum for analysis on at least one occasion. 34 of the 43 (79%) patients who completed the trial were able to provide sputum on one occasion. 24 of the 43 (56%) patients who completed the trial provided sputum for analysis on all three visits. Table 5.6

	40 mg bd	20 mg bd	Placebo
Sputum production of recruited subjects	15/19 (79%)	15/20 (75%)	17/19 (89%)
Sputum production of subjects completing trial	9/13 (69%)	12/15 (80%)	13/15 (87%)
Full sputum set of subjects completing trial	5/13 (39%)	10/15 (67%)	9/15 (60%)

Table 5.6 Sputum production during trial. The majority of patients who were recruited into the trial were able to provide sufficient sputum for analysis at trial entry. The majority of those subjects who completed the trial were able to provide at least one sputum sample for analysis. The proportion of patients who were able to provide sufficient sputum for analysis on all three trial visits was lower in comparison, this drop was most marked in the 40mg bd group.

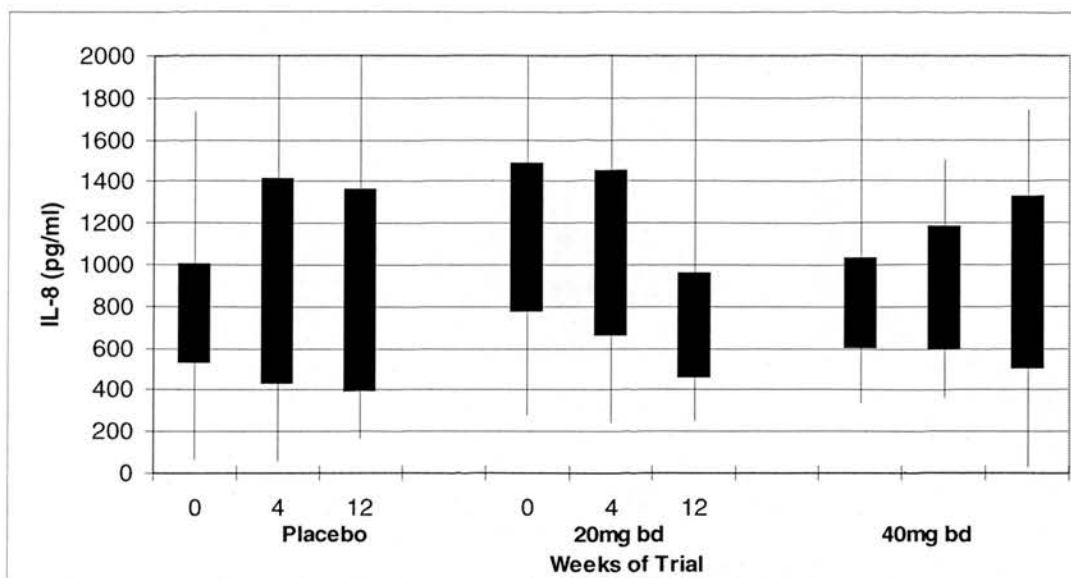
5.15.1 Induced Sputum IL-8

A wide variation was noted in the levels of IL-8 measured in induced sputum prior to trial commencement. There was no significant difference in IL-8 levels measured between the three treatment groups prior to trial commencement.

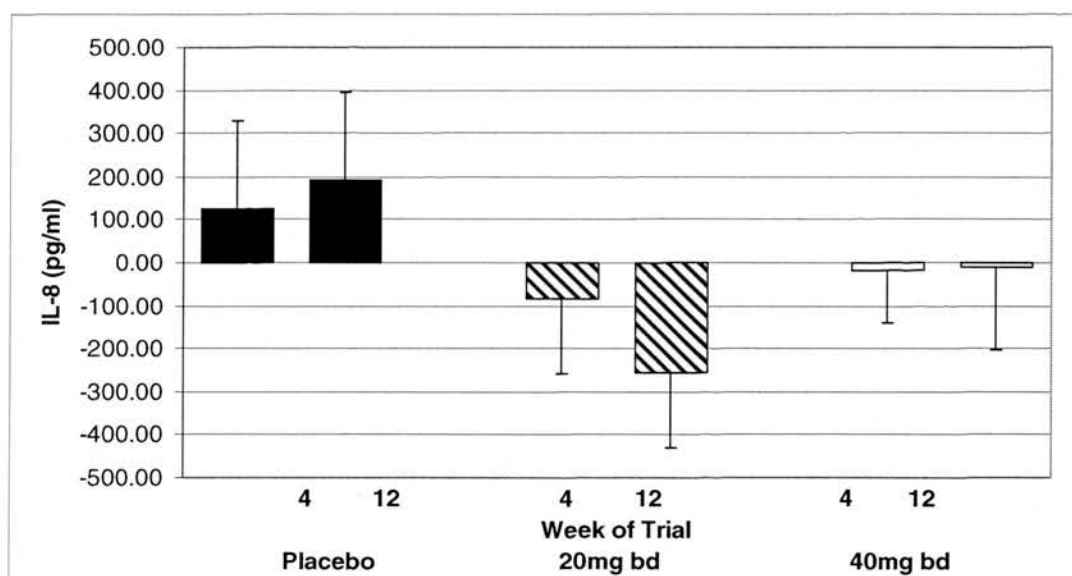
In the 40mg bd group and placebo there were no significant differences in the IL-8 levels measured during the trial.

There was a fall in levels of IL-8 measured in sputum supernatant in the 20mg bd group from  $1108 \pm 597$  pg/ml at trial commencement to  $1026 \pm 561$  pg/ml at week 4 and  $853 \pm 550$  pg/ml at trial completion. Changes from baseline to week 12 of the trial were close to statistical significance,  $p=0.0646$ .

When changes between treatment arms are compared by ANOVA no statistically significant change was demonstrated.



**Figure 5.27 IL-8 Levels in induced sputum during the trial in the three treatment groups.** No statistically significant difference was demonstrated between the three groups during the trial although changes from baseline to week 12 of the trial in the 20mg bd group were close to being significant ( $p=0.06$ )



**Figure 5.28 Change in IL-8 levels in induced sputum from baseline during trial.** Histograms represent the level of IL-8 measured at weeks 4 and 12 of the trial after subtraction of the baseline values. Positive values represent an increase in the level of IL-8 measured during the trial. Black histograms represent placebo, hatched 20mg bd and white 40mg bd. Error bars represent standard error of mean.

5.15.2 Induced Sputum IL-1 $\beta$

There were no significant changes in levels of IL-1 $\beta$  measured during the trial, either within treatment groups or between treatment groups.

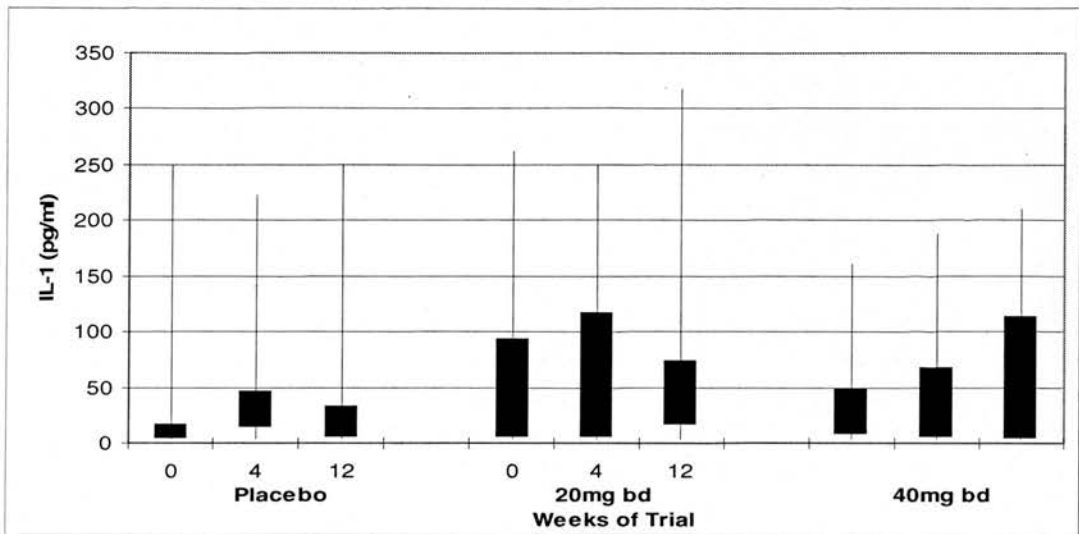


Figure 5.29 IL-1 $\beta$  in induced sputum during the trial in the three treatment groups. No statistically significant difference was demonstrated between the three groups during the trial.

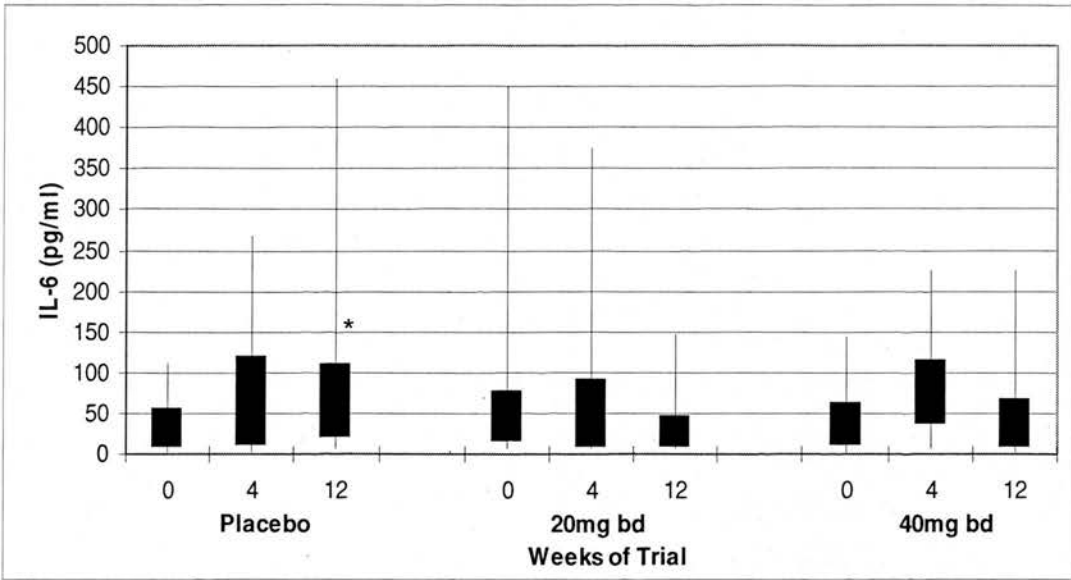


Figure 5.30 Change from baseline levels of induced sputum IL-1 $\beta$  measured during trial. Histograms represent the level of IL-1 $\beta$  measured at weeks 4 and 12 of the trial after subtraction of the baseline levels, positive values represent an increase in the level of IL-1 $\beta$  measured during the trial. Error bars represent standard error of mean.

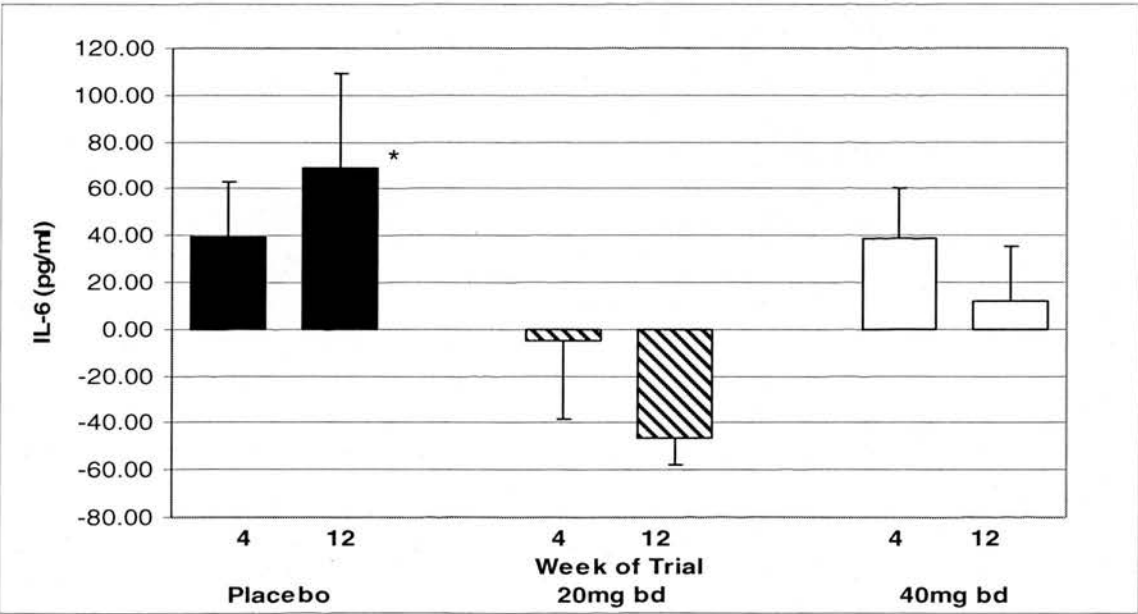
### **5.15.3 Induced Sputum IL-6**

The placebo group demonstrated a statistically significant increase in the levels of IL-6 measured from trial commencement  $34.8 \pm 35.6$  pg/ml to  $103.8 \pm 139.0$  at trial completion,  $p=0.039$ . Mean levels of IL-6 measured in induced sputum in the 20mg bd and 40mg bd groups did not change significantly during the trial. Between group comparison did not show any statistically significant differences.





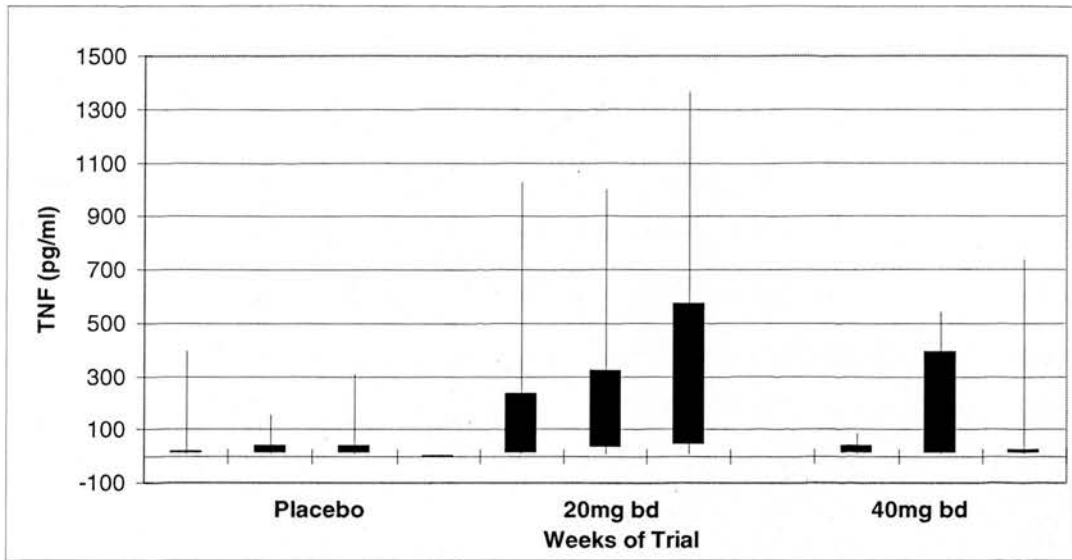
**Figure 5.31 Induced Sputum IL-6 Levels during the trial in the three treatment groups.** A statistically significant increase in the levels of IL-6 measured in the placebo group at trial completion compared with baseline was noted. \* $p < 0.05$  Wilcoxon matched pairs.



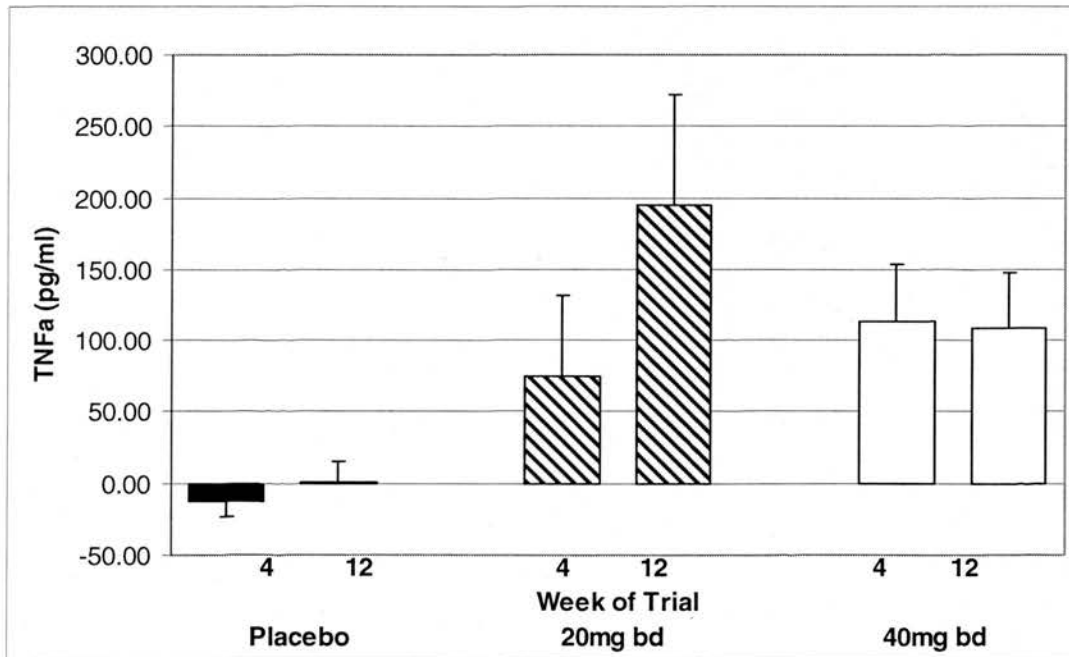
**Figure 5.32 Change from baseline levels of induced sputum IL-6 during trial.** Histograms represent the level of IL-6 measured at weeks 4 and 12 of the trial after subtraction of the baseline levels, positive values represent an increase in the level of IL-6 measured during the trial. Black histograms represent placebo, hatched 20mg bd and white 40mg bd. Error bars represent standard error of mean. A statistically significant increase in the levels of IL-6 measured in the placebo group at trial completion compared with baseline was noted, \* $p < 0.05$ .

#### 5.15.4 Induced Sputum TNF- $\alpha$

A wide variation in the levels of TNF- $\alpha$  was noted prior to trial commencement. A higher average level was present in the 20mg bd group compared with placebo and 40mg bd however this was not statistically significant. The mean TNF $\alpha$  levels in the three groups did not significantly change during the trial.



**Figure 5.33 Induced Sputum TNF- $\alpha$  Levels during the trial in the three treatment groups.** No statistically significant change was noted in the levels of TNF-a measured in induced sputum during the trial in the three groups.

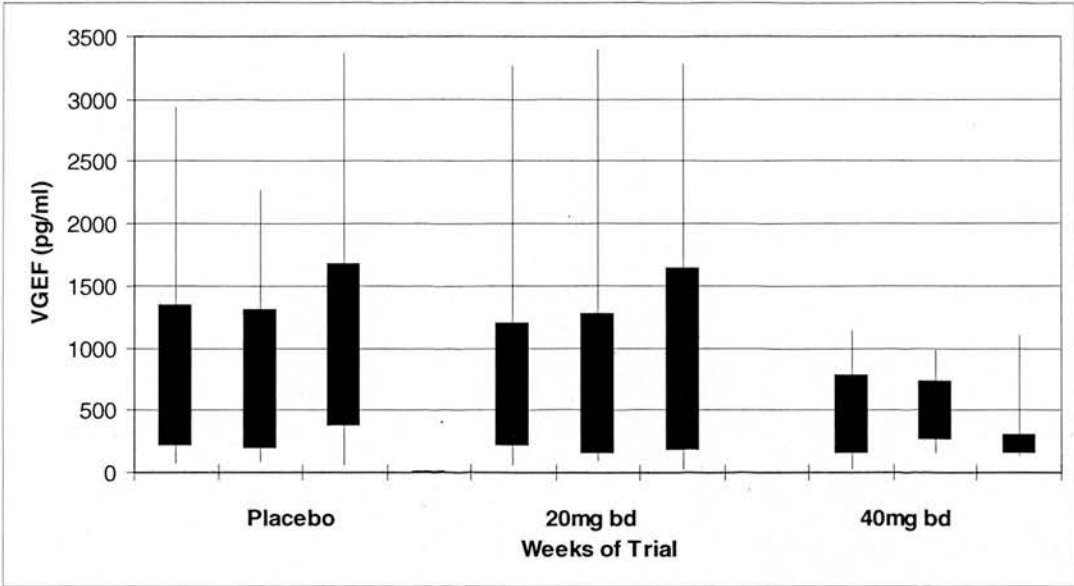


**Figure 5.34 Change from baseline levels of induced sputum TNF- $\alpha$  during trial.** Histograms represent the level of TNF- $\alpha$  measured at weeks 4 and 12 of the trial after subtraction of the baseline levels, positive values represent an increase in the level of TNF-a measured during the trial. Error bars represent standard error of mean. No statistically significant change was noted in the levels of TNF- $\alpha$  measured in induced sputum during the trial in the three groups.

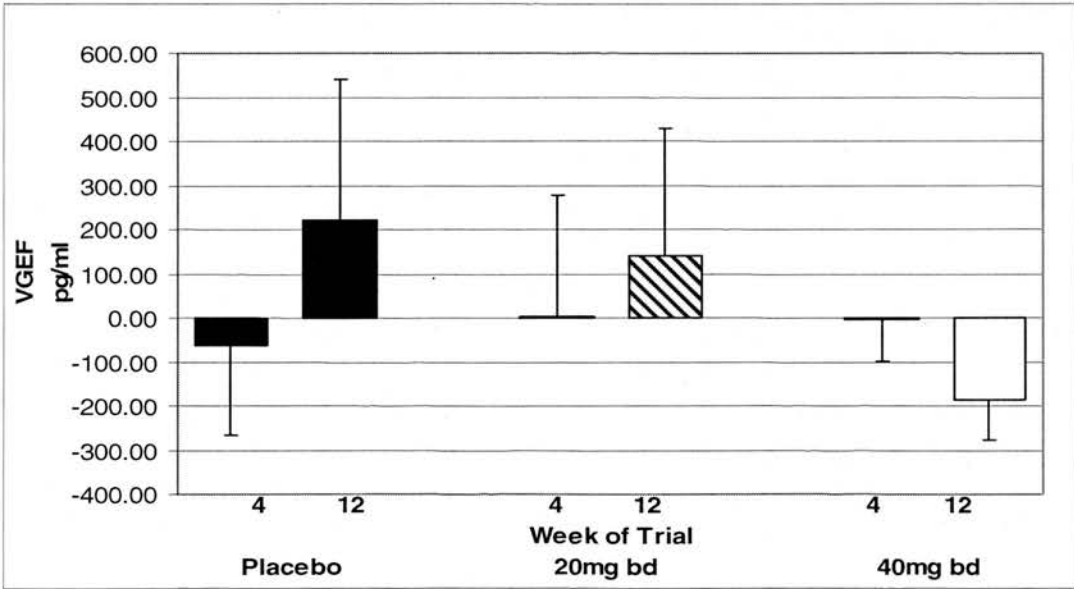
#### **5.15.5 Induced Sputum VEGF**

A wide variation in levels of VEGF measured was noted in all three treatment arms.

The average levels of VEGF in the 40 mg bd group were lower than the 20mg bd and placebo groups at trial commencement. However this was not statistically significant. There was no significant variation in the levels of VEGF measured in induced sputum in the three treatment groups during the trial.



**Figure 5.35 Induced Sputum VEGF Levels during trial in the three treatment groups.** No statistically significant change was noted in the levels of VEGF measured in induced sputum during the trial in the three groups.



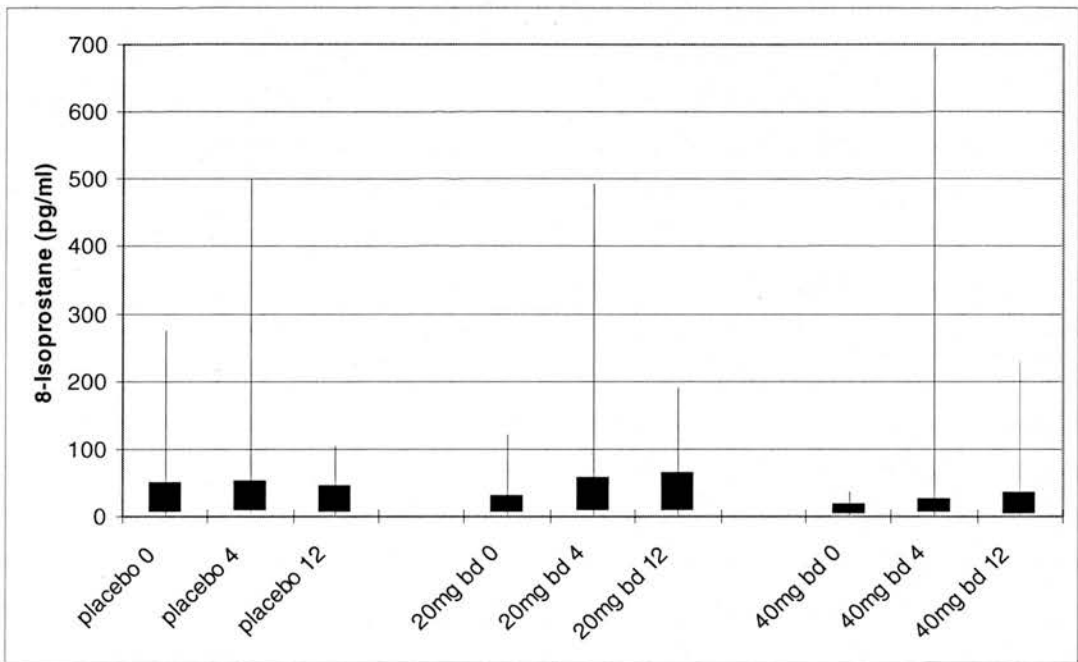
**Figure 5.36 Change from baseline levels of Induced Sputum VEGF during trial.** Columns represent the level of VEGF measured at weeks 4 and 12 of the trial after subtraction of the baseline levels, positive values represent an increase in the level of VEGF measured during the trial. Error bars represent standard error of mean. No statistically significant change was noted in the levels of VEGF measured in induced sputum during the trial in the three groups.

## **5.16 Exhaled Breath Condensate**

Exhaled breath condensate was measured at trial commencement, after 4 weeks of therapy and after 12 weeks of therapy. Three biomarkers of oxidative stress, hydrogen peroxide, 8-Isoprostane and 3-nitrotyrosine were measured along with Leukotriene B4 as a marker of inflammation. A wide variation was seen in levels of all biomarkers measured prior to trial commencement with no significant variation between groups. Treatment with inhaled NAL had no effect on levels of any of the selected exhaled biomarkers during the trial.

5.16.1                      8-Isoprostane

A wide variation was noted in the levels of 8-Isoprostane at baseline for all three groups from 1.5pg/ml to 275pg/ml with no significant variation between the groups. A wide variation in levels of 8-Isoprostane was again noted during the trial in all three treatment groups without any statistically significant changes.



**Figure 5.37 Exhaled breath condensate 8-Isoprostane levels during the trial in the three treatment groups. No statistically significant difference was demonstrated between the three groups during the trial.**

5.16.2                      Hydrogen Peroxide

A wide range of hydrogen peroxide levels measured in EBC at baseline were noted from 0.04µM/ml to 15.43µM/ml. Mean levels of hydrogen peroxide did not vary significantly compared with baseline or placebo in either of the active treatment groups.

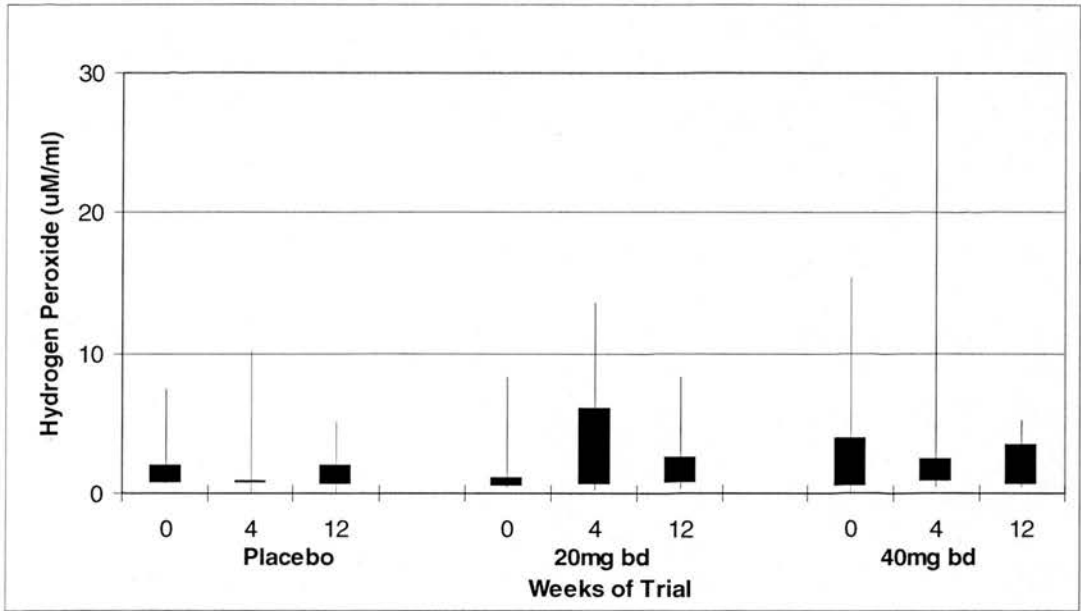


Figure 5.38 Exhaled Breath Condensate Hydrogen Peroxide Levels during the trial in the three treatment groups. No statistically significant difference was demonstrated between the three groups during the trial.



5.16.3 3-Nitrotyrosine

3-Nitrotyrosine was below the detection limits of the assay in two thirds of the EBC samples obtained. There did not appear to be any significant variation within treatment arms in terms of level of nitrotyrosine or numbers detectable, either prior to or during treatment.

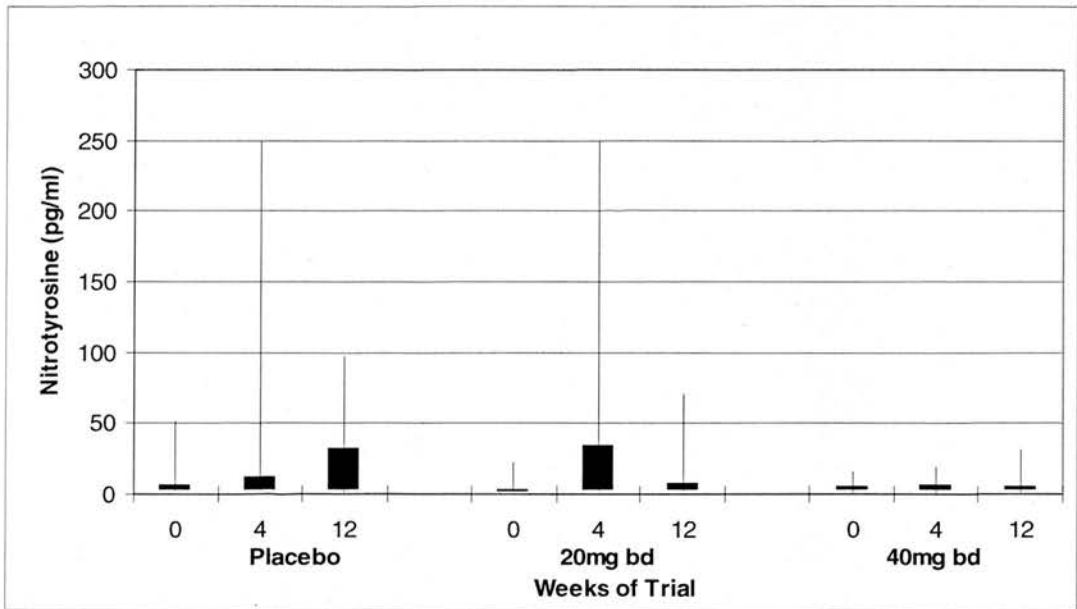


Figure 5.39 Exhaled Breath Condensate Nitrotyrosine Levels during the trial in the three treatment groups. No statistically significant difference was demonstrated between the three groups during the trial.

5.16.4                      Leukotriene B4

As per the project protocol, LTB-4 was last to be analysed. The amount of EBC available from each individual varies. This resulted in only 35 of the 58 trial participants having sufficient EBC available for analysis of LTB-4. Therefore less data was available for this biomarker.

There was no variation in the mean levels of LTB-4 measured in EBC as the trial progressed in either the high dose or low dose NAL groups either compared with baseline or placebo.

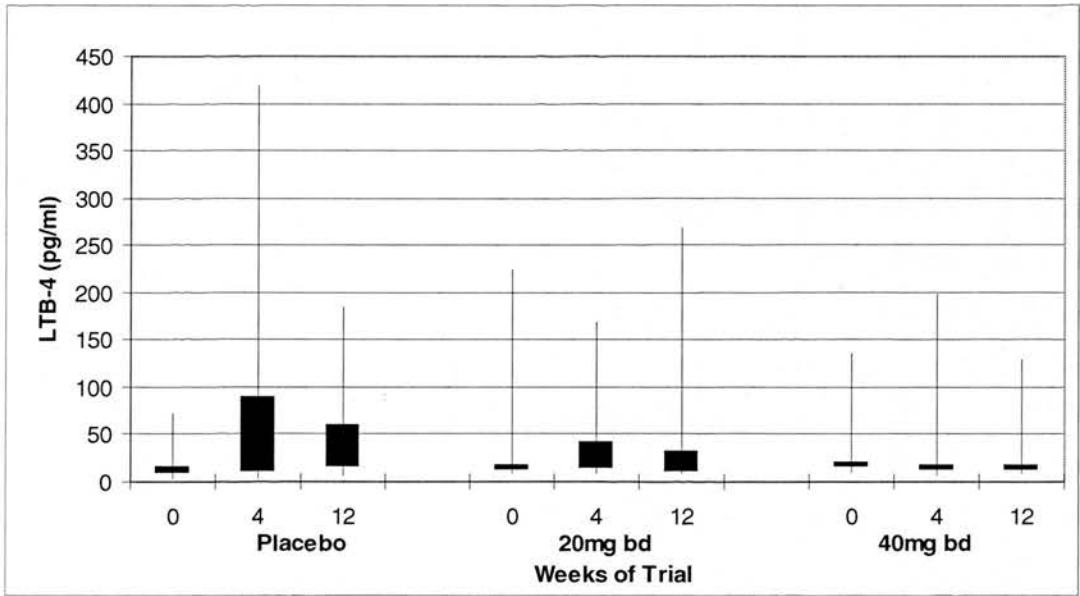


Figure 5.40    Exhaled Breath Condensate LTB-4 Levels during the trial in the three treatment groups. No statistically significant difference was demonstrated between the three groups during the trial.



## 5.17 Conclusions

Inhaled Nacystelyn appeared to be well tolerated during the trial although there was an increase noted in oropharyngeal pain and discomfort in the two treatment arms compared with placebo. Subjects taking the higher dose nacystelyn showed a significant improvement in health status after 4 weeks of trial therapy. However this was not sustained. Low dose nacystelyn demonstrated less substantial but clinically significant improvement, in health status at trial completion along with improvements in diary card reporting of symptoms of cough and breathlessness. A small placebo effect was also observed.

The improvements in health status noted in the two treatment groups were statistically significant as individual data could be paired with baseline levels, strengthening the statistical power. However there were no significant changes when compared to placebo. With the data available the study was insufficiently powered to prove that the changes noted in health status in the treatment arms were statistically different from placebo. A crossover study would have improved the power of the study.

Biomarkers were not effective in demonstrating the action of nacystelyn. A large amount of variation was noted in the majority of biomarkers prior to trial commencement. This may reflect either a wide variation in the amount of oxidative stress and inflammation in the population studied or demonstrates that the use of the non-invasive biomarkers selected is not useful in assessing minor changes in oxidative stress and airways inflammation in COPD.

## **Chapter 6**

### **Discussion and Conclusions**

## **Chapter 6 Discussion and Conclusions**

This chapter discusses the results of the studies described in the previous three chapters and compares these results to previous published work. When possible conclusions are drawn and possible direction of future research identified. The results of each study will be summarised before discussing each study.

## 6.1 Reproducibility of Exhaled Breath Condensate Biomarkers

Levels of both hydrogen peroxide and 8-Isoprostane were measurable in exhaled breath condensate from most patients. The majority of 8-Isoprostane measurements were close to the limit of detection of the assay. There was a statistically significant between-visit correlation in measurement of 8-Isoprostane levels in this study population. Hydrogen peroxide levels were normally distributed although levels were not detectable in a minority of cases. Hydrogen peroxide levels did demonstrate a trend toward correlation between study visits. However this failed to reach statistical significance.

The results of the exhaled breath condensate reproducibility study were similar to previous publications. In the majority of subjects there was either low level of biomarkers measured or none detected. Occasionally a high measurement would be noted. A high level measured on one visit did not guarantee a high level on the second visit. During the trial subjects were assessed at each visit and if there was any clinically significant change in their symptoms or signs then they were withdrawn from the study. These high measurements were therefore independent from any discernable change in their disease process.

There are several factors which may impact on the variability of exhaled breath condensate collection. Previous studies have shown that the time of day of collection(276), the rate of breathing and subsequent volume collected(390), the use of nose clips(391) and saliva contamination(392) all influence the reproducibility of breath condensate.

Other factors which may influence the variability but which have not yet been studied include time to storage at -80°C, current smoking status and time from last cigarette, time of last meal and caffeine intake. Factors related to COPD itself which may influence variability include the FEV<sub>1</sub>, time from last exacerbation and current inhaled therapy. Again there are no published studies regarding these factors.

For the purpose of this study we developed a standard operating procedure following the recommendations of the recent ERS/ATS task force(239), in an attempt to control for some of these factors. The time of day on which EBC was obtained was similar for both study visits. All subjects were asked to wear nose clips and to stop collection if there was a build up of saliva during the collection process. The duration of collection was limited to 10 minutes; however, no attempt was made to limit the rate of breathing. It should be noted that the volume of condensate collected in that time varied from less than 0.5mls to greater than 2mls. Although the concentration of hydrogen peroxide and 8-Isoprostane was measured rather than the absolute amount, the volume collected may have had a significant impact on final results. Further work may be required to assess if controlling the rate of breathing impacts on the reproducibility in EBC.

Several other measures were employed to try and limit the impact which individual's habits and lifestyle may have on the reproducibility. Current smokers were asked to abstain from smoking from the night before the day of collection and all subjects were asked to withhold caffeine intake on the morning of the test. Subjects were not, however, asked to fast prior to the study visit. COPD subjects were asked to withhold inhaled therapy on the day of study visit to attempt to



minimise the effect of these drugs on reproducibility. There is no strong evidence regarding the impact of inhaled therapy or caffeine on EBC measurements. A pragmatic approach was adopted in developing our Standardised Operating Procedure, aiming to minimise the impact of study participation on the individual whilst controlling for factors which may or may not have an impact.

Study entry was deferred if they had experienced an exacerbation in the previous month. It is unclear if 4 weeks is sufficient to allow recovery from an exacerbation. Biernacki et al have shown that 8-Isoprostane levels were elevated at the time of an exacerbation when compared with two weeks and two months after the exacerbation. However, it is unclear from this study if these levels had returned to baseline at that stage(305). Our reproducibility study trial protocol meant that a subject could have serial measurements at four, six and eight weeks after an exacerbation. Biomarker levels may vary as a result of the exacerbation, in turn influencing the repeatability of these measurements.

Thirty seven of forty four (84%) samples obtained in our study had detectable 8-Isoprostane levels. This compares favourably with the previous published reproducibility study on 8-Isoprostane where the authors were only able to measure 8-Isoprostane in 36% of the samples(393). In the majority of cases low levels were detected (below 10pg/ml). Between sample reproducibility of log 8-Isoprostan levels was statistically significant although the concordance correlation co-efficient was low at 0.28.

Hydrogen peroxide measurement was less promising. Twenty four of the 28 samples (86%) obtained for hydrogen peroxide measurement had detectable levels, however between visits concordance was lower than in the 8-Isoprostane study and did not reach statistical significance. This is in keeping with previous EBC studies. Van Beurden et al measured hydrogen peroxide in EBC of 20 subjects with COPD and 20 healthy controls on 5 occasions over a 3 week period. The co-efficient of variation in this study was 45% for the COPD group and 43% for the controls. This study also showed a significant variation in hydrogen peroxide levels when measured at different time points on the same day.(276)

One possible explanation for this is that, whilst both  $H_2O_2$  and 8-Isoprostane are stable end products of oxidative stress, hydrogen peroxide may be metabolised more rapidly than 8-Isoprostane. Previous unpublished study by this group indicated that the acute effects of smoking on hydrogen peroxide levels in EBC returned to baseline 5 hours after smoking. The impact of acute smoking on 8-Isoprostane appeared more complex with elevated levels in some subjects 24 hours after acute smoking. These data indicate that 8-Isoprostane may be present in the lung longer than hydrogen peroxide after excess oxidative stress. Thus over night abstinence may not be sufficient in 8-Isoprostane measurement in EBC.

The between visit variation of hydrogen peroxide measurement was higher in COPD subjects when compared with controls independent of smoking status. This may reflect ongoing acute intermittent oxidative stress, with subsequent variation in hydrogen peroxide production. There were however only 5 subjects in the COPD

sub-group and this result should be interpreted with caution. There was no similar difference between the control subjects and COPD in 8-Isoprostane measurement.

As mentioned earlier, there are other factors which may influence biomarker variability which were not controlled during the trial. Measurement of hydrogen peroxide in 7 atopics and 8 controls demonstrated that mean hydrogen peroxide levels were influenced by the rate of flow of exhaled air, with mean concentration at a flow rate of 48ml/sec  $0.32 \pm 0.03 \mu\text{mol/L}$  compared with  $0.13 \pm 0.02 \mu\text{mol/L}$  at 140ml/sec,  $p < 0.001$ . In this study EBC was collected on different days. The mean co-efficient of variation was high independent of the flow rate, 68% (range 28-193%) at a rate of flow of 140ml/sec and 82% (range 17-930%) at a flow rate of 48ml/sec(394). A further study has shown that the volume of expired air correlates with the volume of EBC collected(390). It may therefore be that rate of breathing has a significant influence on the volume of EBC produced, thus influencing biomarker levels and variability.

Another possible factor influencing the repeatability is sub clinical infection during the trial. Although subjects were screened for significant change in illness between study visits, a minor viral illness between visits may not have been noted. A recent study has shown that hydrogen peroxide levels were acutely elevated in twenty healthy individuals with the common cold  $0.20 \mu\text{mol/L}$ , range 0.03 to  $1.2 \mu\text{mol/L}$  compared with  $0.09 \mu\text{mol/L}$ , range  $< 0.01$  to  $0.4 \mu\text{mol/L}$ , 2 weeks later,  $p = 0.006$ (395). This may be another factor influencing the variability of EBC measurement.

In conclusion, our study corrected for several factors which may influence biomarker variability. The reproducibility of 8-Isoprostane measurement was

improved when compared with previous studies, possibly as a consequence of these corrections. Current smoking, despite overnight abstinence appeared to have a detrimental effect on 8-Isoprostane reproducibility.

Hydrogen peroxide measurement was less reliable, especially in subjects with COPD. Hydrogen peroxide in EBC appears to be a more acute marker of oxidative stress. Minor illness or simply the day to day variation in disease activity may cause significant changes in hydrogen peroxide measurement.

This study indicates that 8-Isoprostane is a more reproducible biomarker when compared with hydrogen peroxide. Further work on this biomarker may further improve the reliability of these measurements. The impact of smoking abstinence acutely on current smokers merits further investigation. The time course of elevated 8-Isoprostane after an exacerbation may also be of interest. The impact of the severity of COPD on reproducibility has not been studied. Subjects with COPD who have very variable disease, with higher exacerbation rates, may have worse reproducibility than those with more stable disease. The disease severity may also affect reproducibility. Finally, another area of study is the role of tidal volume and rate of breathing on the volume of EBC produced and subsequent measurement of biomarkers.





## 6.2 Reproducibility of Induced Sputum Biomarkers

Obtaining induced sputum for analysis in subjects without COPD was problematic, particularly in control non or ex-smokers. The total cell counts obtained and the percentage of macrophages appeared reproducible in the study population. In contrast the percentage of neutrophils and eosinophils counted in sputum did not closely correlate between subject visits.

The cytokines IL-1 $\beta$  and IL-6 were not reproducible in the supernatant of induced sputum. In contrast measurements of levels of IL-8 and VEGF were reproducible during the trial.

Exhaled Breath Condensate was successfully obtained in all subjects on each study visit. This reflects the non-invasive nature of the procedure which was well tolerated by all individuals, even those with severe COPD. In contrast obtaining induced sputum was more problematic, although there were no major side effects from the procedure.

Induced sputum production was low specifically in the control subjects who were not current smokers. Only two of the twenty three subjects (9%) in these groups were able to provide sufficient sputum for analysis on all three occasions. This differs from previous studies. For example Spanevello et al(396) were able to obtain induced sputum in 96 out of 114 healthy non smoking volunteers. It was unclear from the Spanevello paper if the ability to produce induced sputum was a selection criterion. The induction protocol differed from our study. In the Spanevello study 4.5% hypertonic saline was used sequentially for 1,2,4,8 then 16 minutes, a total of 31 minutes maximum or until sputum was obtained. This procedure was tolerated well with no significant drop in FEV<sub>1</sub>(396). In comparison, in our study 3% nebulised saline was administered for 5 minutes, followed by 4% then 5% for five

minutes if subjects failed to provide sputum, a total of 15 minutes maximum. The higher success rate with sputum induction may in part relate to the more prolonged induction protocol.

Sputum induction was more successful in the COPD group in our study. Eighty-six percent of subjects provide sufficient sputum for analysis on all three visits, compared with 95% in a reproducibility study by Brightling et al (314) and 100% in a similar study by Beeh et al (311).

The majority of initial work on reproducibility of induced sputum concentrated on subjects with asthma and cystic fibrosis along with healthy controls. A study of the reproducibility of induced sputum in 31 healthy controls by Purokivi et al showed that 18 were able to produce sputum on two occasions two days apart. The intra class correlation coefficient (ICC) for percentage neutrophils was 0.76, for IL-6 0.57 and for TNF $\alpha$  0.66 (397). These figures are significantly better than our study with ICC of 0.26 for % neutrophils and 0.24 for IL-6. A different protocol was used for sputum induction in the Purokivi study with healthy subjects inhaling 4% saline for 15-20 minutes prior to sputum induction. There was no difference in the sputum processing or sample analysis. There is no comment made in this paper regarding the smoking habits or other subject characteristics. The superior reproducibility in the Purokivi paper may be as a result of prolonged saline administration or may reflect that sampling was performed on two consecutive days.

Measurement of levels of TNF $\alpha$ , IL-8 and neutrophil differential counts in 17 subjects with cystic fibrosis on two days of the same week showed good reproducibility with intra-class correlation 0.93, 0.82 and 0.82 respectively. (398)



Reproducibility of neutrophils differential counts measured in induced sputum of 21 asthmatics on two visits at least two days apart demonstrated an intra class variation of 0.57(309). In a separate study 29 asthmatics had differential cell counts measured on two visits on two consecutive days and showed an intra class correlation of 0.85(399).

Previous studies of reproducibility in COPD demonstrate better reproducibility when compared with our study although results have not been as encouraging as in other airway disease processes. This may in part reflect the diverse disease activity in subjects with COPD.

The superior reproducibility in other studies may be as a result of the selection criteria of the studies. Beeh et al recruited only COPD subjects who were ex-smokers (311), in comparison 8 of the 21 COPD subjects studied in our group were current smokers. Furthermore the COPD subjects in this study had a GOLD level 2 disease, all subjects having FEV<sub>1</sub> between 40% and 70% predicted where as our study recruited patients with FEV<sub>1</sub> ranging from 24% to 80%. The intra-class correlation in the Beeh et al paper was 0.66 for %neutrophils and 0.50 for sputum IL-8, compared with 0.45 for %neutrophils and 0.43 for IL-8 in our study.

Brightling et al were able to obtain sufficient induced sputum for analysis in 116 of 122 subject visits with COPD. The selection criterion for this study was very similar to our study as were the patient characteristics although fewer patients were current smokers(314). Intra class correlation in this study was 0.57 for % neutrophils.

It may be that current smoking habit has a significant effect on the reproducibility of induced sputum, although there are too small numbers in the study groups to allow

further comment on this. Several other factors may influence the reproducibility of induced sputum such as the time from last exacerbation, with other papers having a longer time from last exacerbation than our study, and the use of inhaled corticosteroids. Both the other studies discussed exclusively studied steroid naïve patients.

In conclusion our study has shown that obtaining induced sputum in healthy controls was difficult using our protocol. This may improve with more prolonged sputum induction methods. The sputum induction success rate in subjects with COPD was comparable with previous studies. The reproducibility of %neutrophils and pro-inflammatory cytokines was inferior when compared with other airways diseases. Repeatability in previous studies of COPD was also superior compared with our study, although it does appear that overall induced sputum in COPD is less reliable than in other diseases. Several factors such as time from last exacerbation, current smoking and drug therapy may impact on the reproducibility of the process and merit further study.

Factors relating to the individual subject's disease process, which have not been commented on in previous papers, such as exacerbation frequency, severity of cough, sputum volume and character, degree of airflow limitation and airways lability may all also impact on the reproducibility of induced sputum in COPD. Controlling for some of these factors may improve reproducibility.

### 6.3 Cross Sectional Study of Biomarker Measurement in Exhaled Breath Condensate

None of the four biomarkers studied appear to be useful in differentiation of COPD from patients without respiratory disease. Non respiratory co-morbidity appears to affect measurement of inflammation and oxidative stress in EBC. Breath condensate measurement in current smokers with COPD did not appear to help characterise the disease process. In contrast, in the group of subjects with COPD who were ex-smokers, 8-Isoprostane levels, and to a lesser extent hydrogen peroxide, were related to a variety of health parameters. Prostaglandin E2 and pH measurement did not appear to be of any use in characterising the subjects studied.

Oxidative stress results in generation of free radicals. Cell lipid membranes can be damaged by these free radicals, a process which is known as lipid peroxidation(400). 8-Isoprostane is one of these stable end products and is thought to be biologically active, possibly as a cell mediator(247, 265).

Previous studies have shown that 8-isoprostane is a reliable marker of lipid peroxidation in vitro(249) and in animal models(245, 250, 251). 8-Isoprostane is elevated in the urine of patients with COPD when compared with healthy smoking controls(252). Urine 8-isoprostane correlates inversely with FEV<sub>1</sub> and PaO<sub>2</sub>(266).

Initial studies measuring exhaled breath condensate in COPD were encouraging. Montuschi et al demonstrated increased levels of 8-isoprostane in the EBC of 40 patients with COPD when compared with healthy controls, 12 smokers and 10 non-smokers. 8-Isoprostane levels were elevated in smokers compared with non smokers in the healthy control groups. Furthermore acute smoking led to a rise in 8-Isoprostane in healthy controls(260).

Levels of 8-Isoprostane in EBC of 21 patients with COPD increased during exacerbations, taking up to two months to return to baseline levels(401). A further study confirmed elevated 8-Isoprostane in EBC of 30 patients with COPD during exacerbations. In the same paper 10 subjects with stable COPD were given the antioxidant carbocysteine which resulted in a fall in EBC 8-isoprostane(402). Supplemental oxygen increased levels of 8-Isoprostane in the EBC of 23 patients with COPD, along with 23 healthy subjects(403).

Subsequent published measurements of 8-Isoprostane have been less successful. Kostikas et al have shown that whilst 8-Isoprostane levels were elevated in the EBC of 30 subjects with COPD compared with 10 controls, these measurements did not relate to disease severity, inhaled corticosteroid use or induced sputum differential cell counts, unlike hydrogen peroxide in exhaled breath condensate which demonstrated relationships with these endpoints(404). Van Hoydonck et al collected EBC of 12 smokers three times over a one week period and were only able to measure 8-Isoprostane in 36% of the samples resulting in major concerns over reproducibility of this technique(393).

Our study has again shown that 8-Isoprostane could not always be detected in COPD. 8-Isoprostane was not measured in forty five of the 138 subjects studied (32%). Twenty four of the 138 (17%) had levels below the accepted Limit of Detection of the assay whilst 21 of the 138 (15%) provided samples which were not replicated. The levels of 8-Isoprostane measured were low with only 16 of the 138 subjects having levels greater than 10pg/ml. The proportion of subjects with levels below the limit of detection did not vary between the five populations studied.

No significant difference in mean 8-Isoprostane levels was shown between the groups studied. This may simply reflect the fact that 8-Isoprostane as a biomarker does not differentiate between COPD and controls.

Another possible explanation is that levels of 8-Isoprostane in EBC reflect the degree of systemic and pulmonary oxidative stress independent of the disease process. Whilst subjects with significant pulmonary disease or inflammatory processes were excluded from the trial, subjects with other disease processes including cardiovascular and cerebrovascular disease were not excluded. Indeed only 20 of the 60 (33%) control subjects had no significant co-morbidity. This reflects the fact that we were attempting to recruit control subjects who had a significant smoking history and who were age matched to our COPD population. Therefore these subjects would be expected to have a high incidence of co-morbidity.

8-Isoprostane levels in the control group who had no significant co-morbidity were significantly lower than the subjects with any disease process, including COPD,  $3.4\text{pg/ml} \pm 0.5$  versus  $5.9\text{pg/ml} \pm 0.4$ ,  $p=0.02$ . The other co-morbidities were not strictly categorized and further interpretation of this data is limited.

Current smokers with COPD did not demonstrate any relationship between 8-Isoprostane levels and markers of disease activity. Again this may indicate the limited utility of 8-Isoprostane as a biomarker in COPD. Alternatively, given the relationships demonstrated between ex-smokers with COPD and 8-Isoprostane, it is possible that acute smoking, despite abstaining for 12 hours, may have a greater influence on the level of 8-Isoprostane than the degree of chronic lung disease

activity. The results do indicate however that measurement of 8-Isoprostane in smokers with COPD does not reflect the severity or degree of disease.

Ex smokers with COPD show several relationships with disease activity. 8-Isoprostane levels measured did not display Gaussian distribution. Subjects were therefore divided into quartiles dependant on the level of 8-Isoprostane, including those subjects with 8-Isoprostane levels below the limit of detection. Subjects in the highest quartile had significantly higher exacerbation frequency and lower health status and post bronchodilator percentage predicted FEV<sub>1</sub> when compared with those in the group with the lowest levels of 8-Isoprostane. Non-statistically significant trends were also noted in symptom scores for cough and breathlessness. Levels in quartiles 2 and 3 did not significantly differ with either quartile 1 or 4. The numbers in each quartile were small and these results should be interpreted with caution.

To conclude, in our study, 8-Isoprostane levels in exhaled breath condensate did not vary between subject groups according to the presence or absence of COPD or current smoking status. The control groups in the study had significant co-morbidity which may in part explain this observation.

Ex-smokers with COPD who had high levels of 8-Isoprostane demonstrated higher exacerbation frequency, lower FEV<sub>1</sub> and worse health status compared with subjects with low levels. However the number of subjects in each group was small and a larger, possibly multicentre, study would be required to confirm these initial findings. These results suggest that 8-Isoprostane is of limited use in differentiating COPD from control but may be of some benefit in assessing disease severity in ex-smokers.

Hydrogen peroxide, like 8-Isoprostane, is a product of oxidative stress. Superoxide ion interacts with water to produce this molecule, a process catalysed by superoxide dismutase.  $H_2O_2$  is a stable product and can be measured in exhaled breath condensate.

Emelyanov et al demonstrated elevated levels of hydrogen peroxide in EBC of 70 subjects with asthma compared with 17 healthy controls. Furthermore hydrogen peroxide levels appeared to correlate to  $FEV_1$  and bronchial hyperactivity(272).

Loukides et al were able to demonstrate a similar pattern in bronchiectasis with elevated hydrogen peroxide levels in 37 patients compared with 25 age matched controls with evidence of correlation between hydrogen peroxide levels and airflow limitation(273).

Several trials have shown elevated hydrogen peroxide levels in EBC of patients with COPD. Dekhuijzen et al demonstrated elevated levels in 12 patients with stable COPD compared with 10 healthy controls. Levels were further elevated during exacerbations of COPD(90). Two conflicting trials have recently been published regarding acute changes in exhaled breath condensate hydrogen peroxide levels during exacerbations of COPD.

Gerritsen et al showed that treatment with high dose systemic corticosteroid reduced levels of EBC hydrogen peroxide during the first 7 days of acute exacerbations(274) whilst van Beurden et al did not demonstrate any significant change in hydrogen peroxide levels in the first week after hospitalisation due to lower respiratory tract infection in subjects with COPD(275).



Levels of hydrogen peroxide in COPD patients along with healthy controls demonstrate diurnal variation. Furthermore the coefficient of variation of hydrogen peroxide over 21 days in COPD patients is 45%(276).

Hydrogen peroxide, as with 8-Isoprostane, did not appear to vary significantly between the COPD population and the control group independent of current smoking status. Furthermore ex-smokers with COPD had higher mean levels of hydrogen peroxide when compared with current smokers with COPD. This is despite the COPD current smoking group having worse health status when compared with the ex-smokers. A similar trend was shown in control subjects with ex smokers having higher hydrogen peroxide levels than current smokers.

These results may simply reflect the limited usefulness of hydrogen peroxide measurement in EBC. Previous studies have shown that hydrogen peroxide is stable at -20°C for one month(405). However it is possible that the duration of time that samples were stored in the freezer may have impacted on the reproducibility of these results.

An alternative explanation is that current smokers were asked to abstain from smoking for at least 12 hours prior to trial visits. Inhaled cigarette smoke both contains hydrogen peroxide(406) and generates further hydrogen peroxide in the lung after inhalation(407). A variety of anti-oxidant systems in the lung exist to metabolise  $H_2O_2$  such as catalase and glutathione peroxidase(408). Acute abstinence from cigarette smoke may result in excess activity of anti-oxidant defence compared with levels of oxidative stress; this may result in acute reduction in levels of hydrogen peroxide.



In contrast to 8-Isoprostane, no significant trends were demonstrated between the level of hydrogen peroxide and disease activity in ex smokers with COPD. The most obvious conclusion to draw from this is that  $H_2O_2$  in EBC does not relate to disease activity in COPD.

In summary our study has failed to identify a relationship between the levels of hydrogen peroxide and 8-Isoprostane in exhaled breath condensate and the presence or absence of COPD in current and ex-smokers. 8-Isoprostane levels in ex-smokers with COPD possibly relate to disease severity in these subjects.

## 6.4 Effect of Inhaled Nacystelyn on Subjects with COPD

A variety of end points were measured in the Inhaled Nacystelyn study, including measurement of health status, diary card reporting of symptoms and non-invasive biomarkers. This section will discuss each end point separately.

### 6.4.1 Health Status

Health status assessment as a measurement of disease severity in COPD has developed in recent years. This is in part due to the recognition that COPD is a multi-factorial and systemic disease. Patients who suffer from this disease may have symptoms and signs not directly related to airflow obstruction which can have a significant impact on their health. Examples of this include exacerbation frequency(409, 410), low BMI(41), depression(411) and respiratory muscle weakness(412). Several of these factors are independent risk factors for mortality. It has also been shown that FEV<sub>1</sub>, despite being associated with mortality, does not predict response to treatment with bronchodilator therapy(413), or risk of hospitalisation(414).

The realisation that FEV<sub>1</sub> alone is not a sufficient marker of disease extent and activity in COPD has led to the development of health status assessment tools in COPD.

The St Georges Respiratory Questionnaire is a well validated tool in assessment of health status in chronic respiratory disease. It is reproducible in stable COPD and correlates with exercise capacity, MRC symptom scores, disease activity, anxiety and depression(411),(415, 416) There is a rather poor relationship between FEV<sub>1</sub> and SGRQ in the region of  $r = 0.2$ (417, 418). It has been shown that SGRQ levels

vary with the GOLD stages of COPD(419). SGRQ is also a useful prospective predictor of mortality(411, 420-422) and risk of hospitalisation(414).

Assessment of clinical improvement in health status assessment is problematic(389). Studies using the SGRQ have shown that a change of 4 points is the threshold for clinical improvement, otherwise known as the Minimal Important Difference (MID)(389), although this may vary; subjects with a poor health status may benefit from smaller changes in scores(423). The SGRQ has been used in previous studies to demonstrate changes in health status during exacerbations(424) , bronchodilator(425), long term oxygen therapy(426) and inhaled corticosteroid (ICS) treatment(71). Recent work has shown that use of ICS may reduce the rate of decline in health status(71, 427), the major factor in reducing this rate of decline appears to be the ability of ICS to reduce frequency of exacerbations(428). Health status decline is associated with frequency of exacerbations with more frequent exacerbators having a more rapid rate of decline(428).

Previous studies have shown an improvement in health status with placebo therapy(71, 73, 414). A patient's assessment of health status is subjective. Therefore it is to be expected that placebo treatment has some impact on this. An improvement in health status was demonstrated in the placebo arm of this study. This may simply reflect a placebo effect, however it was noted that a higher number of subjects were not on inhaled corticosteroid at trial commencement and were commenced on ICS at trial entry in the placebo group compared with the 20mg bd group.

Those subjects in the placebo group who received ICS for the first time during the trial show greater improvements in total SGRQ scores when compared with baseline, Table 6.3, although this was not statistically significant. No firm conclusion can be drawn from this due to the small numbers involved but it suggests that part of the reason for the placebo effect may be the addition of an ICS at trial commencement, Figure 6.1.

The St Georges Respiratory Questionnaire has three components; symptoms, activity and impact. The changes shown in health status during the trial relate to different components of the SGRQ in the different study groups. The improvement in health status in the placebo group was due to changes in the impact component at week 4 and the impact and activity components at week 12, with no effect on the symptoms score. In contrast the improvement in the 20mg bd NAL group was due to changes in all three components with symptom scores having the largest effect at both week 4 and trial completion. All three components of the SGRQ improved at week 4 of the trial in the 40mg bd NAL group with all three components returning to baseline at week 12, Figure 6.2.

In conclusion the changes in health status in the placebo group are due to the changes in the impact scores, with activity having a lesser effect, whilst the changes in the active treatment groups are because of improvements in all three components. Overall the changes noted in SGRQ in the treatment groups during the trial were not statistically significant. The mean total SGRQ score for the 58 patients who participated in the trial was  $55.6 \pm 15.3$  points prior to trial commencement. Assuming that the health status distribution was normal and that

three groups were of equal variance, a total of 181 subjects in each group would be required to prove a null hypothesis to the power of 0.8 with a significance of 0.05. This study was therefore significantly underpowered.

As noted in the results section, the 40mg bd treatment group demonstrated significant improvement in health status after 4 weeks of therapy. These improvements returned to pre-treatment levels at trial completion. One possible explanation is that the side effects experienced in the 40mg bd NAL group during the trial were responsible for the deterioration in health status.

Any side effects experienced during the trial were recorded and classified according to system and severity (mild, moderate and severe). The number and severity of side effects experienced during the trial were reviewed according to treatment arm, Table 6.4.

Subjects in the 40mg bd group appeared to experience fewer side effects than in the placebo and 20mg bd groups, although there were more withdrawals in this group, and therefore the total trial duration was shorter, giving less time for subjects to report adverse events. There did appear to be an increased number of moderate or severe adverse events in the 40mg bd group compared with the other arms of the study. However 6 of the moderate or severe events reported in this group relate to one individual who unfortunately was subsequently diagnosed with lung cancer, 3 months after trial completion. Expert opinion from Respiratory Physicians out with the trial was sought and it was felt that this diagnosis was in no way related to the NAL therapy. When this patient was removed from the analysis the number of moderate or severe side effects in the 40mg bd group is similar to placebo.

The total number of recorded side effects did not appear to relate to the treatment arm of the trial. As well as assessing the number of side effects in each group. Further analysis was performed to investigate the relationship between side effects and health status in the 40mg bd NAL group. One possible explanation for the deterioration in health status at trial completion is that this relates to the side effects of Nacystelyn. If this were the case we may expect to see a significant drop in health status in those subjects who experienced side effects, with subjects who tolerated the therapy having a different health status profile, with a significant difference in the SGRQ between these two sub-groups.

Subjects in the 40mg bd group were therefore divided into those who experienced one or more moderate or severe adverse events compared with those who did not. The change in total health status from baseline was compared between the two groups. Analysis was performed in both the intention to treat population and only the subjects who completed the trial.

A total of 9 of the 19 trial subjects reported moderate or severe side effects in the 40mg bd group. Four of these patients withdrew from the trial, 3 within the first 4 weeks. Two subjects in the 40mg bd group withdrew from the trial without reporting any moderate or severe adverse events (Table 6.5).

Subjects who did not experience a moderate or severe side effect during the trial showed larger improvements in health status compared with those who did experience significant side effects. However these differences were not statistically significant. The improvement noted at week 4 in the sub-group of subjects who did not report side effects of the drug returned close to baseline at trial completion.

Similar changes were seen in the sub-group reporting side effects, with improvements at week 4 not sustained at trial completion.

In conclusion, there are several factors indicating that the lack of sustained improvement in health status in the 40mg bd group is not as a result of side effects of Nacystelyn. The number of side effects reported in this group was no different from the other two treatment arms. The subjects in the 40mg bd group who did experience side effects classified as moderate or severe withdrew from the trial within the first 4 weeks and therefore did not impact on the rise and fall noted in health status. Subjects reporting side effects that went on to complete the trial still showed an improvement at week 4 and return to pre - trial levels. Furthermore, the SGRQ deteriorated from week 4 to trial completion in those subjects who did not report adverse events. Another possible explanation is that the observed changes in health status were as a result of the chemical properties of the drug.

There is some evidence that at high concentrations low molecular weight thiols can have a pro-oxidant effect due to autoxidation of sulfhydryl groups(429). This is associated with the Fenton reaction(430) resulting in increased production of superoxide ion and hydrogen peroxide(431).

Sprong et al studied the effect of a variety of doses of N-Acetyl Cysteine, 275mg/kg, 550mg/kg, 950mg/kg in a rat model of LPS - induced lung injury. This study showed that at low dose NAC reduced plasma levels of hydrogen peroxide and improved mortality, whilst high dose resulted in increased plasma levels of hydrogen peroxide and worsened mortality.

Low dose NAC did not appear to alter the amount of total or reduced glutathione compared with control prior to LPS challenge. After administration of LPS there

was a significant increase in the levels of GSH, reduced glutathione, without any major change in the level of total glutathione compared with placebo. This is suggestive that NAC resulted in increased anti-oxidant capacity without increasing the total levels of glutathione.

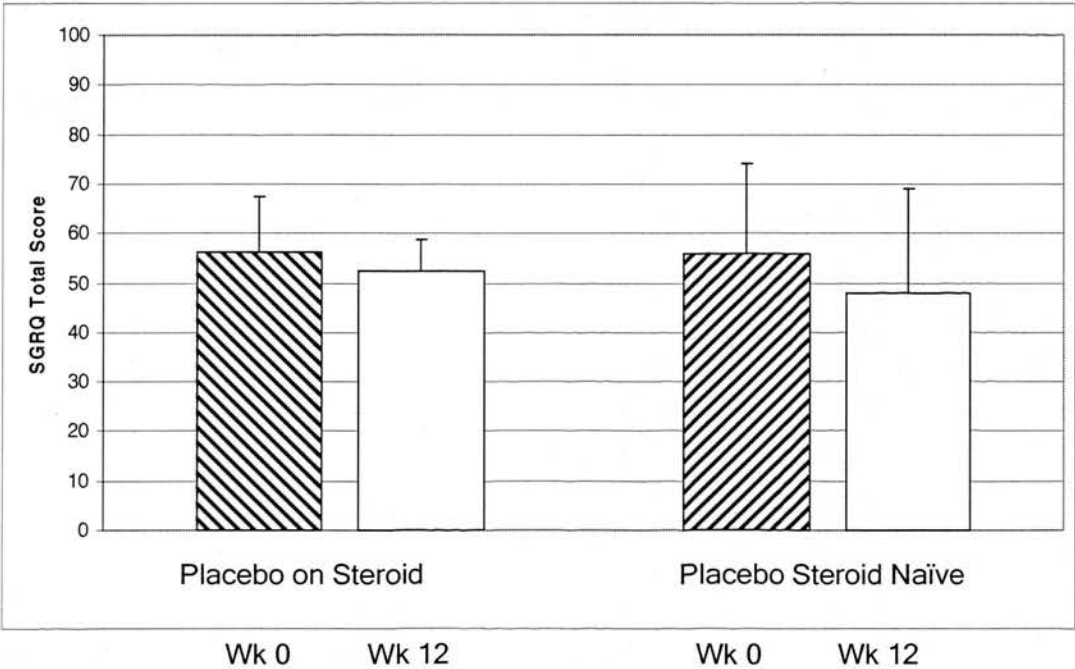
In contrast high dose NAC appears to have resulted in increased levels of total glutathione, without increased GSH, prior to administration of LPS. This indicates that there were increased levels of GSSG, the oxidised form of glutathione, after administration of high dose NAC resulting in reduced anti-oxidant capacity in the lung.

Applying this paper to the NAL study is problematic. There does appear to be a link however, with low dose treatment having a beneficial effect in contrast to high dose.



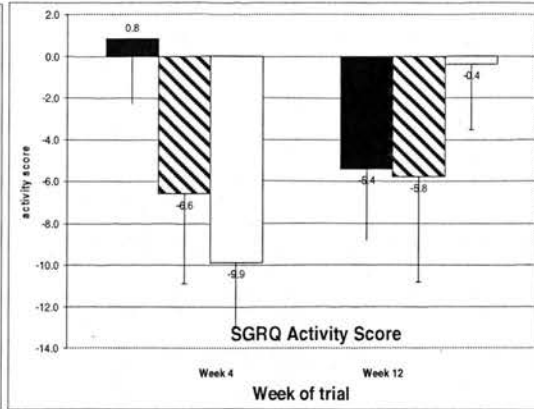
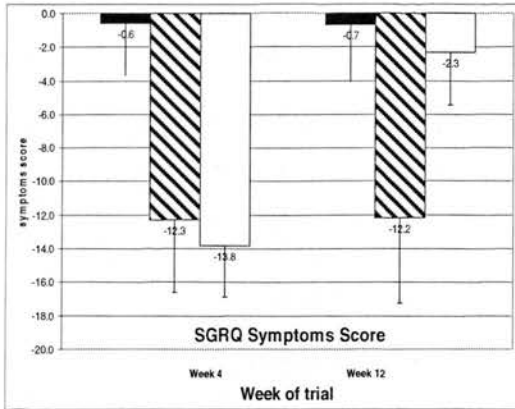
On ICS	Overall	40mg bd	20mg bd	Placebo
Randomised	38/58 66%	9/19 47%	16/20 80%	13/20 65%
Completed	28/44 64%	7/13 54%	12/15 80%	9/16 56%

**Table 6.3** Number of Patients on Inhaled Corticosteroid prior to Trial Recruitment



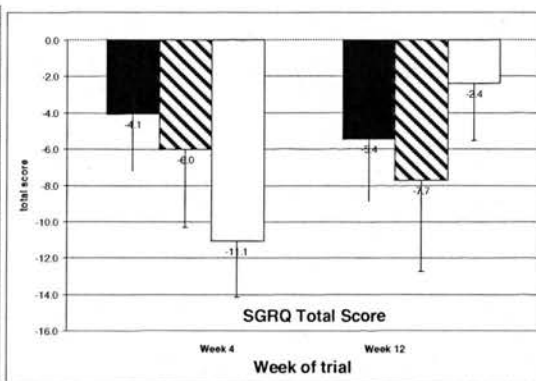
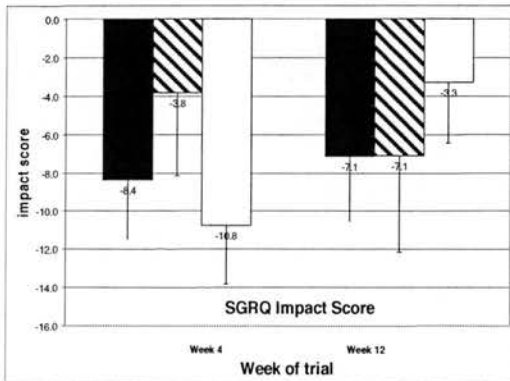
**Figure 6.1** Changes in SGRQ Total Score in Placebo Group dependant on inhaled steroid use prior to study recruitment

Subjects already on inhaled corticosteroid (n=8) had a mean change in total score from 56.1±11.2 at trial commencement to 52.4±6.5 at trial completion, steroid naïve subjects (n=7) had a mean change from 56.0±18.1 to 47.8±21.1.



6.2a

6.2b



6.2c

6.2d

**Figure 6.2 SGRQ Symptom (a), Activity (b), Impact (c) and Total Scores (d).** Change from Baseline at Weeks 4 and 12 of trial for the three treatment groups. Mean SGRQ score was calculated for each treatment group at each of the three study visits; baseline, after 4 weeks and 12 weeks of therapy. The baseline score was then subtracted from the mean score at weeks 4 and 12 of the trial for each treatment group. The three components of the SGRQ are expressed along with the total score. Histograms represent the mean changes from baseline for placebo (black), 20mg bd NAL (hatched) and 40mg bd (white). Error bars express the standard error of mean.

	40mg bd NAL	20mg bd NAL	Placebo
Total SE	54	62	70
Moderate/Severe	27	15	21
No of Patients	9	6	12

**Table 6.4 Side Effects reported during the trial per treatment group**

Change from baseline	Overall	Without Mod/ Severe AE	With Mod/ Severe AE
Week 4	10.4 n=14	12.4 n=8	6.8 n=6
Week 12 or ET	0.2 n=19	2.6 n=10	-2.7 n=9

#### Trial Completion

Week 4	11.1 n=13	13.0 n=8	8.0 n=5
Week 12	2.4 n=13	4.0 n=8	-0.2 n=5

**Table 6.5 Changes in St Georges Respiratory Questionnaire during trial in 40mg bd group. Group subdivided dependant on presence or absence of side effects**

### 6.4.2 Diary Cards

Compliance with the diary cards appeared good with all subjects returning completed diary cards. Diary card reporting of the number of days with no or mild breathlessness in the 20mg bd group significantly improved compared with placebo, sustained improvements from baseline in the 20mg bd group were also noted in breathlessness, cough and number of days with mild or no cough. When compared with the St Georges Respiratory Questionnaire there was no similar improvement in diary card reporting of symptoms early in the trial in the 40mg bd. Not every subject in the 20 mg bd group improved during the trial. Eight of the 15 trial completers demonstrated significant improvement in both breathlessness and cough scores. Subgroup analysis was performed to identify if there were any characteristics specific to these subjects.

Subjects in the 20mg bd group whose breathlessness improved during the trial were more likely to have a high BMI ( $26.1 \pm 2.9$  versus  $21.4 \pm 2.8$ ,  $p=0.009$ ) and had a trend towards worse health status scores ( $60.1 \pm 15.2$  versus  $44.9 \pm 18.6$ ,  $p=0.09$ ). These subjects also had a higher baseline breathlessness score ( $2.4 \pm 0.7$  versus  $1.1 \pm 0.9$ ,  $p=0.02$ ).

Subjects in the 20mg bd group who show a 1 point or more improvement in cough score at trial completion compared with baseline had a higher baseline cough score ( $2.0 \pm 0.6$  versus  $1.0 \pm 0.3$ ,  $p=0.009$ ).

No other biomarker endpoint predicted those subjects who would improve their cough score. Baseline SGRQ did not predict which subjects would improve. Baseline FEV<sub>1</sub> did not predict improvement in cough or SOB score.

These data suggest that there may be characteristics specific to subjects who respond to Nacystelyn. The variable response noted may relate to different phenotypes, subjects with high BMI and cough scores are traditionally viewed as having chronic bronchitis as their predominate pathology. Low BMI and low cough score is more suggestive of emphysema. The limited data presented regarding the subject characteristics are by no means conclusive. However it may be speculated that subjects with chronic bronchitis (and mucus hyper secretion) are more likely to respond to this therapy.

### **6.4.3 Exacerbation Frequency**

The exacerbation frequency of those subjects taking 20mg bd NAL was significantly lower than in the placebo group. The exacerbation frequency in the placebo arm of the study appeared high. However it does appear that close monitoring of subjects with COPD demonstrates that these patients frequently experience exacerbations of their disease without seeking medical attention. Seemungal et al monitored 101 patients over two and a half years. These patients experienced 504 exacerbations, over half of these, 254, were not directly reported to the study doctors but were found on review of diary cards and spirometric measurement(432) A further study from the same group demonstrated an exacerbation frequency of 2.5 per year in moderate to severe COPD with more than half of these exacerbations not reported to medical staff(433). The exacerbation frequency, based on reporting of symptoms, in the placebo arm of the Nacystelyn study was equivalent to 2.3 per year.

#### 6.4.4 Measurement of Plasma Biomarkers

Thiobarbituric Acid Reactive Substances (TBARS) as an assessment of oxidative stress should be interpreted with caution(434). Previous work has shown that certain chemicals, including malondialdehyde and amino acids, when present in biological fluid can react with thiobarbituric acid leading to a falsely high level of TBARS(435). However, TBARS do appear to have some discriminatory properties in COPD. Higher levels of plasma TBARS have been shown in subjects with COPD compared with control subjects, which is most marked after exercise(296, 436). Oral N- acetyl cysteine has been shown to attenuate the increase in plasma TBARS seen after exercise(296).

Plasma TBARS levels during the trial were significantly higher prior to trial commencement when compared with measurements during the trial, independent of the trial group. This may relate to the time that samples were stored in the freezer, with MDA in frozen serum reacting and producing more TBARS. Another possible explanation for this relates to the trial protocol.

Baseline plasma measurement was obtained at trial screening, at which stage subjects not previously on steroids were commenced on inhaled corticosteroids and those subjects already on ICS were standardised to Fluticasone 250mcg bd. Plasma was sampled at this stage of the trial as the results of U+Es, LFTs and FBC were required prior to randomisation. Plasma sampling was not repeated until week 4 of the trial. Therefore there was no baseline measurement available after initiation of ICS but before starting Nacystelyn.

Previous studies have shown that inhaled corticosteroids can have some effect on markers of oxidative stress in COPD. Lower levels of nitrotyrosine and inducible nitric oxide synthetase in induced sputum have been measured in subjects with

COPD after treatment with ICS(303). COPD patients taking ICS have lower levels of exhaled ethane compared with COPD steroid naïve patients(288). In contrast other markers of oxidative stress in the exhaled breath condensate of patients with COPD do not appear to be altered. Steroid treatment has failed to demonstrate any effect on levels of 8-Isoprostane, hydrogen peroxide, LTB-4, PGE2, PGD2 and PGF 2 $\alpha$ (263, 304-307).

Sub-group analysis was performed to investigate if inhaled corticosteroid use prior to trial entry affected the levels of plasma TBARS measured when compared with steroid naïve subjects. TBARS levels of steroid naïve subjects were statistically higher compared with those already taking a form of inhaled steroid,  $3.91\mu\text{mol/L} \pm 3.96$  versus  $1.34\mu\text{mol/L} \pm 2.43$ ,  $p=0.035$ . This finding suggests that taking regular ICS may lower the levels of plasma TBARS. However this is a relatively small sample population and further investigation of this finding is required.

Further interpretation of this result is problematic. There was no further measurement prior to randomisation. Those subjects who were previously steroid naïve were commenced on two new inhaled therapies, fluticasone at week -4 then nacystelyn or placebo at week 0, both of which potentially could have an impact on plasma levels of oxidative stress, prior to second assessment of levels at week 4.

Further analysis was performed dividing the three treatment arms into those subjects who were steroid naïve and those who were on steroid prior to trial entry. The changes in TBARS levels in the placebo group were analysed in an attempt to identify the impact of ICS alone. TBARS levels in the treatment groups who were already on ICS was also analysed to identify the impact of Nacystelyn alone.

Levels of plasma TBARS in the steroid naïve placebo group (n=7) fell from 3.3  $\mu\text{mol/L} \pm 4.0$  at baseline to 1.3  $\mu\text{mol/L} \pm 2.0$  at trial completion,  $p=0.12$ . TBARS levels in the placebo group who were on ICS prior to trial entry were lower at baseline (0.68  $\mu\text{mol/L} \pm 1.1$ ,  $p=0.09$ ) when compared with the steroid naïve subjects and did not change significantly during the trial.

Subsequent analysis was performed on those patients receiving active treatment who had previously been on steroid. Removing the steroid naïve patients from this analysis was based on the assumption that initiation of ICS may have an effect on levels of TBARS.

A trend towards reduction in plasma TBARS levels is noted in the 20mg bd group, 0.8  $\mu\text{mol/L} \pm 1.4$  at trial commencement versus 0.5  $\mu\text{mol/L} \pm 0.7$  at week 12,  $p=0.074$ . A further trend towards lower TBARS levels was noted in the 40mg bd group at week 4 compared with baseline, again not statistically significant.

In conclusion, TBARS levels were significantly elevated at baseline when compared with time points during the trial. This may in part relate to a significant proportion of the trial subjects not being on ICS prior to trial entry. The subjects in the placebo group who were steroid naïve had a trend towards a fall in the TBARS level after ICS treatment suggesting a possible effect of ICS on systemic oxidative stress.

Further investigation of the role of inhaled corticosteroid on plasma measurement of oxidative stress is merited. Specific points of investigation include the effect of ICS on TBARS levels after initiation of treatment. Another area of interest is the relationship between TBARS levels and exacerbation frequency. ICS have been shown to reduce exacerbation frequency(437). It may therefore be that the lower



level of TBARS seen in the steroid group relates to either a lower exacerbation rate or the time from last exacerbation

	Baseline	4	12
40mg bd NAL Steroid Naïve n=6	3.4±3.3	0.6±0.6	1.2±1.8
20mg bd NAL Steroid Naïve n=3	5.9±6.2	0.2±0.2	3.7±6.0
Placebo Steroid Naïve n=7	3.3±4.0	2.7±4.2	1.3±2.0
40mg bd NAL Steroid n=7	3.3±4.7	1.1±2.3	1.9±3.5
20mg bd NAL Steroid n=12	0.8±1.4	0.5±0.7	0.5±0.7
Placebo Steroid n=8	0.7±1.1	0.7±1.0	0.3±0.2

**Table 6.6 Levels of plasma Thiobarburic Acid Reactive Substances (µmol/L) in each treatment arm subdivided by inhaled corticosteroid use prior to trial entry**

### 6.4.5 Measurement of Induced Sputum Biomarkers

Induced sputum collection was successful in the majority of patients at trial commencement (81%) but only 56% of the patients who completed the trial were able to provide sufficient sputum for analysis on all three occasions. As discussed earlier in this chapter the proportion of subjects able to produce sputum in our studies is lower than in previous published trials. Factors which may relate to this reduced sputum production are also discussed above. Along with these methodological issues affecting sputum production, it should be noted that theoretically Nacystelyn has mucolytic properties(385). Diary card analysis demonstrated trends towards reduced sputum production in the study groups. Therefore the low sputum yield during the trial may in part be a drug effect.

A trend towards a reduction in induced sputum IL-8 was shown during the trial. Although these changes were not statistically significant, the trends would be in keeping with the study hypothesis. As mentioned earlier glutathione acts as a major scavenger for free radicals, hydrogen peroxide and lipid peroxides ((438, 439). Increasing the amount of Glutathione in the ELF may potentially result in reduced amounts of ROS, reduced activation of the NFkB pathway and reduced IL-8 synthesis.

Further analysis of the induced IL-8 levels was performed, comparing IL-8 to health status, diary card response and plasma TBARS. There was no direct correlation between health status and IL-8 levels or with changes in health status and changes in IL-8 levels. A similar picture was seen with the diary cards with no direct relationship between the changes in diary card reporting of symptoms and levels of IL-8 in induced sputum. No obvious trend was seen between TBARS and IL-8.

IL-1 $\beta$  appears to correlate with IL-8 in induced sputum. This would be in keeping with previous studies indicating that IL-1 $\beta$  stimulates production of IL-8(440). No significant difference was seen in the levels of IL-1 $\beta$  in the three treatment arms during the trial or any correlation with markers of health status and systemic oxidative stress.

Unlike IL-8, there was no change in levels of IL-6 measured in the two active treatment arms during the study. IL-6 levels in the placebo group increased during the trial. This may reflect the high frequency of exacerbations in this group, over 50% of subjects on placebo experienced an exacerbation during the trial compared with 20% in the 20mg bd arm. Previous studies have demonstrated that levels of IL-6 are elevated during exacerbations of COPD compared with stable conditions. Furthermore subjects with high exacerbation frequency have higher mean levels of IL-6 when stable compared with subjects with low exacerbation frequency(321).

Induced sputum IL-6 levels measured in the placebo group are significantly higher in those subjects who experienced an exacerbation during the trial (187.8pg/ml $\pm$ 263.1) compared with those who did not have an exacerbation of their airways disease (33.7pg/ml $\pm$ 39.7),  $p=0.013$ , Figure 6.3.

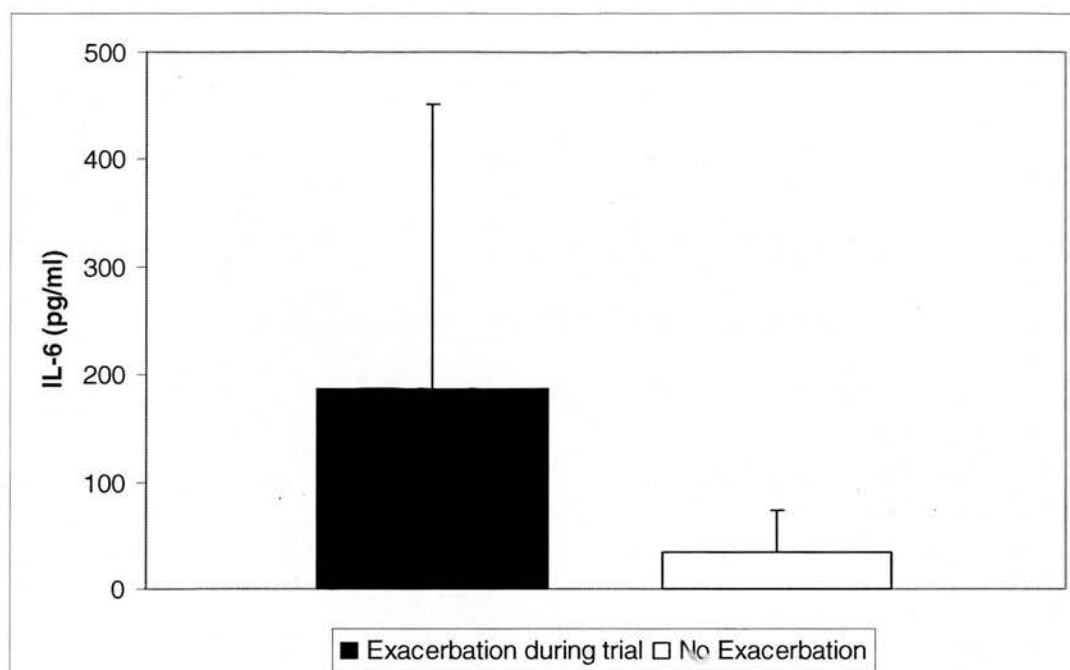
The increase in levels of IL-6 noted during the placebo arm of the trial may therefore be explained by the level of exacerbations of COPD seen in this arm of the trial. This may reflect the frequency of exacerbations in this trial group. Another possible explanation is that increased levels of oxidative stress and subsequent airways inflammation are in themselves a trigger for exacerbations of COPD. The lack of increase in levels of IL-6, and subsequent exacerbations of COPD, in the

treatment arms could be explained by reduced oxidative stress due to treatment by inhaled anti-oxidant.

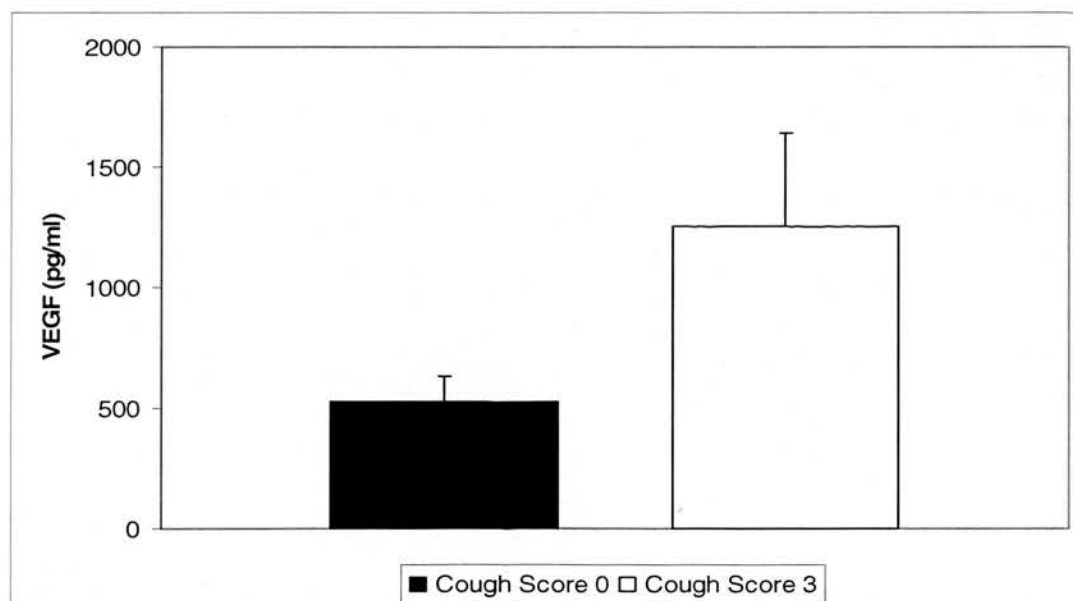
No correlation was shown between sputum IL-6 and markers of health status and systemic oxidative stress.

TNF- $\alpha$  appeared to produce a relatively weak signal, and was not detected, or at the limit of detection in the lowest quartile of each group. This may reflect our use of DTT in sputum processing, previous work has demonstrated that DTT can interfere with TNF $\alpha$  analysis(441).

Previous work has shown that VEGF levels are dependant on the phenotype of COPD with higher levels seen in chronic bronchitis compared with emphysema. Strict characterisation of the phenotype of COPD was not undertaken in this study. Diary card recording did allow some characterisation of the levels of cough experienced each week. Scores ranged from 0, no cough, to 3, cough throughout day. When the levels of VEGF measured are compared with the average diary card symptoms of cough on the week prior to visit, subjects who scored 0 on the week of sputum collection had statistically significant lower levels of VEGF (425.5pg/ml $\pm$ 296) compared with those who scored 3, (1255.1pg/ml $\pm$ 1099), although it should be noted that there were small numbers in each group (n=8 in both groups) Figure 6.4. As previously noted, subjects with high cough scores were more likely demonstrate improvements in diary card reporting of cough and breathlessness. There was however no significant relationship shown between improving diary card symptom scores and VEGF levels.



**Figure 6.3 Mean IL-6 levels in induced sputum of placebo group during trial dependant on exacerbation episode during trial.** Induced sputum IL-6 levels measured in the placebo group are significantly higher in those subjects who experienced an exacerbation (black bar) during the trial ( $187.8\text{pg/ml} \pm 263.1$ ) compared with those who did not have an exacerbation (white bar) of there airways disease ( $33.7\text{pg/ml} \pm 39.7$ ),  $p=0.013$ .



**Figure 6.4 Mean VEGF levels in induced sputum dependant on cough score.** Mean Induced sputum VEGF levels in trial subjects with a cough score of 0 (black bar) are lower than in subjects with a cough score of 3 (white bar), ( $425.5\text{pg/ml} \pm 296$  versus  $1255.1\text{pg/ml} \pm 1099$ ),  $p=0.058$ .

#### 6.4.6 Exhaled Breath Condensate

Exhaled breath condensate collection in the Nacystelyn trial added little to the study hypothesis. The biomarkers studied did not show any difference in levels between the treatment and placebo arms. Although this may simply reflect the limited utility of EBC biomarker measurement in COPD, several methodological issues may have contributed.

Chapter 4 of this thesis discusses the reproducibility of hydrogen peroxide and 8-Isoprostane measurement in EBC. Reproducibility over three visits in a four week trial was reasonable with a concordance correlation co-efficient of 0.66, (95% confidence interval 0.34 to 0.84,  $p=0.0007$ ) for 8-Isoprostane and 0.48 (95% confidence interval 0.00 to 0.77,  $p=0.057$ ) for hydrogen peroxide.

In contrast the concordance correlation coefficient between visit 0 and visit 12 in the placebo arm of the trial was -0.07 for 8-Isoprostane with 95% confidence interval -0.43 to 0.30,  $p=0.77$  and -0.20 (-0.73 to 0.50,  $p=0.60$ ) for hydrogen peroxide.

Whilst this variation may reflect the nature of COPD with day to day variability in symptoms the correlation between visits is significantly less reliable than in the Chapter 4 study. Similar subjects participated in the two trials and both the study protocols and analysis techniques were identical. Several methodological issues specific to this trial may have contributed to this variability. Exhaled breath condensate was stored at  $-80^{\circ}\text{C}$  until individual subjects completed the trial so that the three samples obtained for each individual were analysed with the same reagents and standards. Sample analysis was performed in batches of at least 12 individuals, again in an attempt to reduce between subject variations by using the

same reagents. As a result first visit samples had been stored at -80°C for at least 3 months, many for longer than this.

When the levels of 8-isoprostane for all subjects, independent of treatment arm, are compared with the stage of the trial, baseline levels are significantly lower than week 4 of the trial, with a trend towards being lower than at trial completion. The level at week 0 is on average 26.3pg/ml ( $\pm 45.7$ ), 89.8 ( $\pm 172.1$ ) at week 4 of the trial, and 40.6 ( $\pm 56.3$ ) at week 12. This suggests that the duration of storage at -80°C may have an impact on levels of 8-isoprostane measured, with some degradation of samples as time progresses. A further factor relating to the variability relates to the study protocol allowing one exacerbation during the trial. If subjects experienced an exacerbation the study protocol was still followed and EBC collection could only be deferred by one week. The duration of increased oxidative stress in the lung after an exacerbation is not known. It is therefore possible that increased levels of oxidative stress measured in EBC could be as a result of a recent exacerbation. Subjects had to be exacerbation free for four weeks prior to trial entry. However, the lower levels measured at trial entry, independent of study group, compared with weeks 4 and 12 may be in part as a result of this minimum time from last exacerbation.

In conclusion, 8-Isoprostane was not a reliable or stable marker in this trial with significant variation in levels seen in the placebo arm. This does not allow further interpretation of the data therefore it neither proves nor disproves the hypothesis that inhaled antioxidant may have an effect on lipid peroxidation in the lung. Similar high levels of variation were seen in the placebo arm with hydrogen peroxide, nitrotyrosine and leukotriene B4. There were similar trends towards decreasing



levels dependant on the duration that the sample was in the freezer; however these were not statistically significant.

### 6.4.7 Summary

In conclusion inhaled Nacystelyn (NAL) was well tolerated during the trial. A significant improvement in health status was shown after 4 weeks of high dose NAL. However this was not sustained during the trial. The lack of sustained effect does not appear to relate to side effects from the drug and may be as a result of the impact of the drug on the redox balance in the lung. Further study of the medium to long term effect of inhaled NAL on redox status in humans is required.

Low dose NAL appeared to show a sustained effect, both in terms of health status measures and diary card reporting of symptoms. The improvement was statistically significant when compared with baseline, but not compared with placebo. A larger powered study would be required to demonstrate any improvement compared with placebo.

Diary Card reporting of breathlessness demonstrated an improvement in symptoms in the 20 mg bd group when compared with placebo, with no significant change shown in the 40mg bd group

Biomarker measurement in EBC during the trial did not mirror improvement in reporting of symptoms and health status. Prolonged storage of breath condensate may have an impact on levels measured and merits further investigation as does the duration of increased oxidative stress in the lung after an exacerbation.

Induced sputum changes were suggestive of treatment benefit in the 20mg bd group; again study numbers were not sufficient for confirmation of these trends.

Plasma measurement of Thiobarburic Acid Reactive Substances suggests that

regular inhaled corticosteroid may impact on levels of systemic oxidative stress, again this finding merits further investigation.

## 6.5 Conclusion

This thesis has demonstrated that some biomarkers of oxidative stress and airways inflammation, such as 8-Isoprostane, Hydrogen Peroxide and Prostaglandin E2, can be measured in exhaled breath condensate, both in subjects with COPD and healthy controls. Other biomarkers, including nitrotyrosine, leukotriene B4, Myeloperoxidase, Tumour Necrosis Factor  $\alpha$  and Interleukins 6, 8 and 10, could not be reliably measured in EBC.

Biomarker measurements in exhaled breath condensate were on or around the limit of detection for the assays used.

The log of 8-Isoprostane was reproducible in the trial although the correlation coefficient was low. Hydrogen peroxide in EBC was close to statistical significance. Acute smoking and time from last exacerbation of COPD as well as methodological issues may have had an influence on this

The reproducibility in induced sputum again may relate to methodological issues in our study, although it should be noted that there was a wider range of disease severity in our study compared to previous along with current smokers being included in our study.

None of the biomarkers which were measured in EBC varied significantly between subjects with COPD and controls. This is part may reflect the high level of co-morbidity in our control groups.

Quartile analysis of 8-Isoprostane levels in ex-smokers with COPD suggested a possible relation ship with health status, symptom score and exacerbation frequency. This suggests a role in future assessment of COPD.

Treatment with an inhaled antioxidant had no significant impact on the identified biomarkers in EBC. Interleukin-6 levels did appear to rise in induced sputum in the placebo group, possibly reflecting the increased exacerbation frequency in the placebo arm when compared with the 20mg bd group.

High dose inhaled Nacystelyn (40mg bd) did have a significant impact on health status compared to baseline after 4 weeks of treatment. However this returned to pre treatment levels by the end of the study. Health status and diary cad symptom scores improved in the lower dose Nacystelyn (20mg bd) group although the study was not significantly powered to detect differences from placebo.

Several future areas of study have been identified in this thesis. Further analysis of several methodological issues identified in exhaled breath condensate collection may improve the reproducibility of this technique, specifically in 8-Isoprostane collection, which shows some promise in assessing disease activity in ex-smokers with COPD. The impact of non respiratory disease on EBC levels may also be of interest.

This thesis has also shown a different clinical response to differing doses of inhaled anti oxidant. This may reflect higher doses disturbing the oxidant-antioxidant balance in the lung. Again this area merits further investigation.

In summary this thesis has been able to identify some reproducible biomarkers of oxidative stress and airways inflammation in COPD and relate these to disease severity. These biomarkers were not able to differentiate COPD from controls. Inhaled anti-oxidant had some clinical response, which may reflect the mucolytic properties of this chemical, but had no effect on biomarkers of oxidative stress.

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# Appendix

## Appendix 2.1 EXHALED BREATH CONDENSATE COLLECTION

### Pre-test (subject)

1. Measure exhaled carbon monoxide.

### Pre-test (equipment)

1. Turn on Condensate Machine 30 minutes before sampling.

### Test

1. Select clean mouth piece, collection tube, collection port, saliva trap and breathing valve.
2. Ask subject to rinse mouth with water prior to test.
3. Place nose clips on subject.
4. Ask subject to perform normal tidal breathing into mouth piece (whilst seated) for 10-15 minutes. If there is a build up of saliva collect in tissue. Subjects may come off the mouthpiece for up to 1 minute to rest (can occur maximally twice), as long as total collection time is 10-15 minutes.
5. Place sample on ice immediately after collection.
6. Pipette as 0.25ml aliquots into autoclaved Eppendorf tubes and freeze at  $-80^{\circ}\text{C}$ .
7. Freeze within 30 minutes of collection.

## Appendix 2.2 INDUCED SPUTUM COLLECTION

### Pre procedure

1. Subject should have a post bronchodilator (Salbutamol 2.5mg via nebuliser) FEV<sub>1</sub> recording to be used as a baseline.
2. If baseline FEV<sub>1</sub> < 1 litre a medical practitioner should be consulted prior to commencement.

### Equipment

1. Spirometer (Vitalograph).
2. Salbutamol nebulules 2.5mg.
3. Sonix 2000 nebulizer unit.
4. Hypertonic saline - 3%, 4%, 5%.
5. Polystyrene 100ml sputum collection pot.

### Procedure

1. Ask subject to rinse mouth with water and gargle vigorously.
2. COPD patients should be asked to expectorate any spontaneous sputum and this should be discarded.
3. Fill Sonix 2000 nebulizer with 5ml sterile hypertonic saline and ask subject to inhale the mist through the mouth piece for 5 minutes. The subject should discontinue inhalation on development of any significant symptomology such as wheeze, breathlessness or lightheadedness. The subject should be supervised at all times by a nurse or doctor during this period.
4. On completion of above, ask to blow their nose and rinse their mouth with water.
5. Ask subject to attempt sputum expectoration into a 100ml collection pot.
6. Any sample produced should be examined for presence of sputum plugs and to assess if an adequate sample has been obtained.
7. Record a further FEV<sub>1</sub> reading on the subject.
8. If no suitable sample has been produced, and the subjects FEV<sub>1</sub> is > 90% pre procedure, then repeat steps 3) to 7) using incremental doses of hypertonic saline up until completion of the 5% saline or a total nebulised period of 15 minutes. Subsequent attempts at expectoration should be into the same sputum pot in order to obtain a cumulative volume. The pot should be kept refrigerated between attempted collections.

9. In event of FEV<sub>1</sub> falling to 80%-89% of baseline then continue to nebulise at the same concentration of hypertonic saline up until such time as:
  - a. an adequate sputum sample has been produced
  - b. a total nebulized period of 15 minutes (3x5 minute periods with FEV<sub>1</sub> checked between)
  - c. FEV<sub>1</sub> returns to > 90% of baseline - resume steps 3) to 7)
  - d. FEV<sub>1</sub> falls to < 80% of baseline - cease procedure
  - e. patient becomes uncomfortable or significantly symptomatic - cease procedure
10. If FEV<sub>1</sub> falls to <80% of baseline procedure should be abandoned.

#### **Additional Safety Points**

1. A trained medical practitioner should be contactable during the procedure and able to attend promptly should an adverse event occur.
2. On completion of the procedure subjects should be given salbutamol (2.5mg) if FEV<sub>1</sub> drops to <90% baseline or the subject feels significantly more breathless or wheezy.
3. Subjects should remain in the unit until FEV<sub>1</sub> is >90% of baseline unless the attending medical practitioner instructs otherwise.



## Appendix 2.3 Induced Sputum Processing

### PLUG REMOVAL

1. Weigh sputum and pot, and note down reading in the whole sputum/total cell on the sputum form so that 13.5052g is rounded and written as 13505.
2. Empty sputum into a Petri dish.
3. Weigh empty pot and note as before but in the whole sputum/tara cell.
4. Weigh empty falcon tube and note reading as before but in the selected sputum/tara cell.
5. Separate out plugs and weigh falcon tube containing plugs and note down in the selected sputum/total cell.
6. Subtract whole sputum/tara from whole sputum total and note in whole sputum /total-tara cell.
7. Subtract selected sputum/tara from selected sputum total and note in selected sputum/total-tara cell, then note selected sputum/total-tara x4 in DTT volume, note that answer will be in micro litres so to make into ml convert 4234 to 4.234 etc.

### SPUTOLYSIS

1. Freshly dilute 1% DTT by ten (0.1%DTT) using PBS(-).
2. Add volume of 0.1% DTT as noted in sputolysin volume.
3. Break up plugs and place tube on ice on rocker for 15mins.
4. Add volume of PBS equivalent to that added of 0.1% DTT.
5. Filter with gauze.

### CLEANING

1. Centrifuge: 10mins, 4°C, 1200rpm.
2. Separate the supernatant, label and put on ice.
3. Add PBS to re-suspend pellet (cells) and centrifuge: 10mins, 4°C, 1000rpm then put on ice.
4. Centrifuge the supernatant: 8mins, 4°C, 2000rpm then put on ice.

## CELL COUNTS

1. Remove and throw out supernatant from the cleaned cells, and re-suspend in PBS at a volume that is more than 250 $\mu$ l and approximate density of  $1 \times 10^6$  cells/ml. Note suspension volume on sputum form in  $\mu$ l.
2. Dilute 10 $\mu$ l of cells with either 90 $\mu$ l Trypan blue (1:100) or with 10 $\mu$ l Trypan blue (1:2) depending on how cloudy the cell suspension is and note dilution on the sputum form.
3. Add to haemocytometer and count how many dead (blue), alive (clear) and squamous (spread out) cells are in the four corners of large squares and note cell count on the sputum form.
4. Calculate viability by:  $\text{alive}/(\text{dead} + \text{alive}) \times 100$ .
5. Calculate squamous by:  $\text{squamous}/(\text{dead} + \text{alive} + \text{squamous}) \times 100$ .
6. Calculate cells/ml by multiplying the alive cells by 25,000 (dilution 1:100) or by 5,000 (1:2) and note on sputum form in multiples of a million cells ( $z \times 10^6$ ).
7. Calculate total cell count by dividing cells/ml by 1000 and multiplying by the suspension volume, note on sputum form as multiples of a million cells ( $z \times 10^6$ ).

## CYTOSPINS

1. Ideal cell concentration for a cytospin  $1 \times 10^6$  cells/ml, dilute where appropriate with PBS by using the cells/ml calculation as a guide.
2. Add 100 $\mu$ l cell suspension to the funnel (with slide and filter) and spin for 3mins at 300rpm.
3. Leave to dry.
4. Aliquot supernatant into min of 0.5ml and max 9 aliquots.
5. Store in -20°C freezer until end of day, then relocate to -80°C
4. Fix and stain (3mins each stain) rinse with water/
5. Leave to dry.
6. Add DPX and cover-slip when ready to count.
7. Do differential cell count and store noting slide box location on cell count sheet.

## Appendix 2.4 Venesection

1. Each venesection will involve collection of approximately 20 ml of blood. Whole blood samples will be collected for subsequent separation into plasma and serum constituents.
2. At each venesection, a volume of 9 ml of blood will be taken in a lithium-heparin treated Vacutainer tube and 5ml blood in an EDTA Vacutainer tube.
3. The blood will be gently mixed after collection and then centrifuged at 2000 rpm for 20 min at 4°C.
4. Lithium heparin samples will be aliquoted into appropriately labelled tubes as follows:
  - a. aliquots of 600µl into polypropylene tubes (for subsequent measurement of C-reactive protein)..
  - b. 3 aliquots of 500µl/aliquot into autoclaved Eppendorf tubes.
5. EDTA samples will be aliquoted (3 aliquots of 500µl/aliquot) into appropriately labelled autoclaved Eppendorf tubes.
6. All plasma aliquots will be stored at -80°C.
7. At each venesection, a volume of 5 ml of blood will be taken in an anti-coagulant free tube.
8. Samples will be allowed to clot at room temperature (20-25°C) for 30 min until the clot just begins to retract.
9. Once clotted, tubes can be kept for up to 2 hours in a refrigerator at 4°C or in an ice-water bath before processing.
10. Spin the samples in a centrifuge at 2000 rpm for 10 min, 4°C.
11. Transfer the cell-free serum to a collection tube.
12. Serum will be stored as 2 x 500µl aliquots in appropriately labelled autoclaved Eppendorf tubes.
13. All serum samples will be stored at -80°C.

## Appendix 2.5

### Spirometry

Spirometry will be recorded using a Vitalograph spirometer.

1. Prior to use the machine should be calibrated.

2. Enter patient details as follows:

Age: age in years;

Height: heights in cms;

Sex: Press MALE or FEM;

Ethnic origin: Press CAUCASIAN or NON-CAUCASIAN.

3. To define which parameters are to be printed, press SETUP and choose option

2. Scroll up/down list and select the parameters you require before pressing

CONTINUE to return to main menu. Graphs to be printed can also be selected from the SETUP menu.

4. To measure FEV<sub>1</sub> select option 2 from the main menu. Axes are drawn and

PERFORM TEST appears. The subject should be instructed to breathe in as deeply as possible and then to blow into the mouthpiece as hard and as fast as possible, and to continue breathing out until their lungs are empty.

5. As the subject finishes the first breath, press END TEST. When the subject is ready press RETEST. At least 3 recordings should be made. On the second and subsequent breaths, BEST TEST VARIATION will appear. This should be kept within  $\pm 5\%$ . If it is not, extra tests should be done.

6. When the test is over, press EXIT to return to the main menu. The results can then be viewed in a number of ways, including printout option.

7. For reversibility testing (COPD patients at screen only in the current study), press 4 (set post-mode) from the main menu and answer YES to ARE YOU SURE. POST will light up on the keypad. Post-bronchodilator results can now be recorded and results viewed in the same way as before. Administer 2.5mg of nebulised salbutamol then repeat 4-6 15-30 minutes after administration of salbutamol.
8. After tests on each subject have been completed and printouts obtained, press 6 from the main menu to clear results before the next subject.
9. All printouts should be labelled with time of test, patient initials, study subject number and should be initialled by the operator.

## Appendix 2.6 8-Isoprostane Protocol

Commercially available kit from Cayman Chemical

### Contents

1	96 well coated EIA Plates.
2	Tracer.
3	8 Isoprostane Standard.
4	EIA Buffer Concentrate.
5	Wash Buffer Concentrate.
6	Tween 20.
7	Antiserum.
8	Plastic film.
9	Ellman's reagent.
10	Mltrapure Water.

### Preparation

1. EIA Buffer Dilute one vial (4) with 90mL  $\mu$ ltrapure water.
2. Wash Buffer Dilute one vial (5) with 2 L  $\mu$ ltrapure water  
Add 1ml Tween 20 (5a).
3. Standard Dilute one vial (3) with 900 $\mu$ l  $\mu$ ltrapure water (conc 5ng/ml).
4. 8 standards EIA Buffer, serial dilutions, from 500pg/ml.
5. Tracer Dilute one vial (2) with 6ml  $\mu$ ltrapure water if 96 well kit, and 30ml if 480 well kit.
6. Anti Serum Dilute one vial (6) with 6ml  $\mu$ ltrapure water if 96 well kit, and 30ml if 480 well kit.

### Assay Day 1

1. Each plate must have blank (BL), non specific binding (NSB), maximum binding (Bo) and total activity (TA) in duplicate.
2. Wash plates once with wash buffer.
3. Add 100µl EIA buffer to NSB wells.
4. Add 50µl EIA buffer to Bo wells.
5. Add 50µl of sample/ standard to each well.
6. Add 50µl of tracer to each well except TA and BL RETAIN 10µl of tracer for day 2.
7. Add 50µl of Antiserum to each well except TA. NSB and BL.

Cover plate with plastic film and incubate for 18 hours at room temp.

### Assay Day 2

1. Reconstitute one vial of Ellman's (8) with 20ml ultrapure water (must use within 24hrs, protect from sunlight).
2. Empty and wash wells (100µlx3).
3. Add 200µl Ellman's to each well.
4. Add 5µl of tracer to TA wells.
5. Cover with film, shake in dark for 60-90 mins.
6. Read plate when Bo absorbance is between 0.3 and 0.8.
7. Read at 405-420nm.

## 2.7 Hydrogen Peroxide Assay

### Solutions

#### Citrate Buffer 0.42M, pH 3.8

1. Make 0.418M Sodium Citrate (3.075g in 25ml dH<sub>2</sub>O)
2. Make 0.629M Citric Acid (3.0265g in 25ml dH<sub>2</sub>O)
3. Add 20ml of Citric Acid to the Sodium Citrate and monitor the pH. Titrate the last 5ml of the Citric Acid into the Sodium Citrate until it reaches pH 3.8. If all the Citric Acid has been titrated yet the buffer is still not at pH 3.8 then adjust using 10M Citric Acid.

NB: it is very important that the pH of the citrate buffer is accurate as the sample may precipitate at a pH outside 3.8.

4. Store at 4°C and use within 4 weeks

#### Horseradish Peroxidase (HRP) - Make up fresh

1. Dissolve 1mg of HRP (222 Puropurogallin Units) in 1ml dH<sub>2</sub>O.
2. Keep in darkness at 4°C and use within 24hrs.

### TMB

1. Dissolve 10mg TMB in 1ml of DMSO (4.2M).
2. Keep in darkness at 4°C. Note that DMSO freezes at 4°C so allow time to thaw prior to use.

#### Stop Solution (1 M H<sub>2</sub>SO<sub>4</sub>)

1. Add 2ml of 18N (2N = 1M) H<sub>2</sub>SO<sub>4</sub> to 16ml of dH<sub>2</sub>O (total volume - 18ml) on ice.
2. Store at room temperature.



## Protocol

1. Firstly check the molarity of the stock 30%  $\text{H}_2\text{O}_2$  solution. This does not need to be done every time the assay is run but should be performed every month or so. To determine the molarity of  $\text{H}_2\text{O}_2$ , prepare the following solutions:
2. 25 $\mu\text{l}$  of stock  $\text{H}_2\text{O}_2$  in 5ml of 1x PBS = 5ml of  $\text{H}_2\text{O}_2$  at a 1:200 dilution.
3. 100 $\mu\text{l}$  of solution A + 900 $\mu\text{l}$   $\text{dH}_2\text{O}$  = 1ml of solution A at a 1:10 dilution
4. Measure the  $\text{OD}_{280}$  of solution B (blank against  $\text{dH}_2\text{O}$ ) in a **QUARTZ** cuvette and determine the concentration of solution A using the following calculation:
5. Concentration of Solution A (mM) =  $\frac{\text{OD}_{280}}{0.01} \times 30 \times 10$
6. The result is in mM so multiply the result by 200 to give the concentration of the stock  $\text{H}_2\text{O}_2$  in mM and then divide by 1000 to give the result in M. Using the result calculate the correct dilution factor to give an accurate set of standards.
7. Make up the standard curve to the following concentrations diluting firstly down to a 1M concentration, then to 10mM before diluting to the concentration of the top standard. This is the serially diluted to give an 8 point standard curve across the range 100 - 0.78 $\mu\text{M}$ .
8. Mix 90 $\mu\text{l}$  of Citrate Buffer, 10 $\mu\text{l}$  of TMB and 10 $\mu\text{l}$  of HRP together for each well. This can either be done in the plate or made up (9ml of Citrate buffer, 1ml of TMB and 1ml of HRP mixed per plate) and 110 $\mu\text{l}$  added to each well.
9. Add 100 $\mu\text{l}$  of standards/ samples to each well as per plate plan including a blank containing 100 $\mu\text{l}$  of  $\text{dH}_2\text{O}$ .
10. Mix and then leave for 20 minutes at room temperature.
11. After 20 minutes add 50 $\mu\text{l}$  of stop solution (1M  $\text{H}_2\text{SO}_4$ ) and mix until fully stopped. If some wells remain blue after the stop solution has been added then wait and it will turn yellow eventually.
12. Read at 450nm.

<i>Std</i>	<i>Molarity</i>	<i>Dilution Factor</i>	<i>Dilution</i>	<i>Final Volume</i>
1	1M	1:10.2	100µl of 10.2M H <sub>2</sub> O <sub>2</sub> in 920µl dH <sub>2</sub> O	1020µl
2	10mM	1:100	10µl of std 1 in 990µl dH <sub>2</sub> O	990µl
3	100µM	1:100	10µl of std 2 in 990µl dH <sub>2</sub> O	990µl
4	50µM	1:2	500µl of std 3 in 500µl dH <sub>2</sub> O	500µl
5	25µM	1:2	500µl of std 4 in 500µl dH <sub>2</sub> O	500µl
6	12.5µM	1:2	500µl of std 5 in 500µl dH <sub>2</sub> O	500µl
7	6.25µM	1:2	500µl of std 6 in 500µl dH <sub>2</sub> O	500µl
8	3.13µM	1:2	500µl of std 7 in 500µl dH <sub>2</sub> O	500µl
9	1.56µM	1:2	500µl of std 8 in 500µl dH <sub>2</sub> O	500µl
10	0.78µM	1:2	500µl of std 9 in 500µl dH <sub>2</sub> O	1000µl

NB: Bold text signifies dilutions made to reach the top standard from the stock solution and are NOT actual standards.

## 2.8 Nitrotyrosine Assay

### Preparation

Bring all reagents to room temperature prior to use.

1. Dilution Buffer - mix the 10ml supplied with 100ml dw (distilled water)
2. Nitrotyrosine Ab - reconstitute vial with 1ml of dw (can be stored at 2-8°C for 1 month or at -70°C for longer)
3. Streptavidin Peroxoxidase reconstitute vial with 1ml dH<sub>2</sub>O. Add 23ml of working dilution.
4. Washing buffer - mix the 20ml supplied with 780 ml of working dilution buffer

### Preparing Standard Curves

1. Reconstitute Nitrotyrosine standard with dw as instructed on the label. This gives a 4.5μM solution.
2. Label tubes 1-8.
3. Add 300μl of working dilution buffer to tubes 1-7 and 500μl to tube 8.
4. Add 150μl of Nitrotyrosine standard to tube1, mix, and continue to serially dilute 150μl down to tube 7. Do not add any to tube 8 as this is the blank.

Tube	1	2	3	4	5	6	7	8
Conc nM	1500	500	166.7	55.6	18.5	6.6	2.1	0

## Assay Procedure

1. Prepare samples by defrosting on ice. If using plasma samples, dilute samples 10:1 with working buffer dilution, e.g. 30 $\mu$ l with 270 $\mu$ l.
2. Add 100 $\mu$ l of standard/sample to each well.
3. Cover and incubate for 1 hour at room temperature.
4. Wash 4 times with wash buffer.
5. Add 100 $\mu$ l Nitrotyrosine Ab.
6. Wash further 4 times.
7. Add 100 $\mu$ l Streptavidin Peroxidase.
8. Cover plate and incubate for 1 hour at room temperature.
9. Prepare TMB Substate 15 minutes prior to completion of above incubation. This is done by mixing TMB Substrate, with Substrate Buffer, and Substrate Dilution Buffer in a ratio of 1:1:2. ( Make up the required volume only which is calculated by multiplying the number of wells by 100 $\mu$ l. Store in darkness by covering in tin-foil before placing in refrigerator. Use within 15 minutes.
10. Wash wells further 4 times.
11. Add 100 $\mu$ l of freshly prepared TMB substrate.
12. Cover plate and surround with tin-foil to thus incubating wells in darkness for the next 30 minutes.
13. Add and mix 100 $\mu$ l Stop solution.
14. Read at 450nm

## Calculation

Note that the blank absorbance should be less than 0.4

1. Plot net absorbance against Log to base 10 concentrations.
2. Derive concentration by applying Power 10 function to Log concentration.
3. If samples diluted, correct by multiplying concentration by 10.

## 2.9 IL-8 ELISA Protocol

### Solutions (enough for one plate)

- PBS with calcium and magnesium (PBS+)
- DMEM with pen/strep + L-glut.
- Wash Buffer (add 250µl of Tween 20 to 500ml of PBS+)
- Blocking Buffer (5% sucrose and 1% BSA in PBS+) Add 1g of sucrose and 0.2g of BSA to 20ml of PBS+ (make fresh - will require stirring).
- Reagent Diluent (0.1% BSA and 0.05% Tween20 in 1X TBS). Dilute 10ml of 10X TBS with 90ml of distilled water. Take 30ml of this solution and add 0.03g of BSA and 15µl of Tween20.
- TMB (add 10mg to 1ml of DMSO) (Keep in dark!!).
- Substrate Buffer (in fridge - see bottle for recipe).
- 1M H<sub>2</sub>SO<sub>4</sub> (sulphuric acid).

### Coating the Plate ( Day before the ELISA)

Dilute 60µl of capture antibody in 10.6ml of PBS+. Add 100µl to each well, then cover the plate and incubate overnight at room temp.

### ELISA Procedure - all incubations at room temp.

1. Remove the capture antibody and wash the wells 3x with 100µl of wash buffer.
2. Add 100µl of blocking buffer to each well, cover, then incubate for 60-90 minutes on the plate shaker.
3. Make standards (20µl standard in 1.8 ml media = 1000 pg/ml stock)  
Standards from 800 - 0 pg/ml.
4. Remove the buffer and wash the plate 3x with 100µl of wash buffer.
5. Add 100µl of standards and samples to each well, cover and incubate for 60-120 minutes on the plate shaker.
6. Remove and wash the plate 3x with 100µl of wash buffer.
7. Dilute 60µl of the detection antibody with 10.6ml of reagent diluent. Add 100µl per well, cover and incubate on the plate shaker for 60-90 minutes.
8. Remove and wash the plate 3x with 100µl of wash buffer.
9. Dilute 50µl of streptavidin-HRP (supplied with the kit in the fridge) in 10ml of reagent diluent. Add 100µl per well, cover and incubate on the plate shaker with foil on top for 20 minutes.

10. Remove and wash the plate 3x with 100µl of wash buffer.
11. Mix 100µl of TMB solution and 5µl of hydrogen peroxide in 10ml of substrate buffer. Add 100µl per well and place in the dark to allow colour development.  
WARNING - the colour develops extremely quickly!!!
12. Add 50µl of sulphuric acid to each well to stop the reaction. Read with a test wavelength of 450nm and a reference wavelength of 570nm.

Similar protocols used for measurement of IL-1 $\beta$ , IL-6, TNF- $\alpha$  and VEGF.

## 2.10 C-Reactive Protein Assay

### Contents of Kit

1. One Holder with precoated strips.
2. ELISA wash buffer concentrate.
3. POD Antibody, Rabbit-anti-CRP, peroxidase labelled.
4. Calibrators.
5. Control.
6. Dilution Buffer.
7. TMB Substrate.
8. ELISA stop solution.

### Materials/ Equipment Required and not supplied

1. Centrifuge.
2. Vortex Mixer.
3. Microplate (45onm).
4. Distilled Water.
5. Absorbent paper.

### Preparation and Storage

Wash Buffer dilute wash buffer concentrate with aqua dest 1:10. Crystals may have formed due to high salt concentrate- these should be dissolved in water bath at 37°C before dilution of concentrate. Diluted solutions at 2-8°C stable for one month.

POD Labelled Antibody. Dilute to 1:100 in ELISA wash buffer. The antibody is not stable after dilution.

### Specimen Collection and Preparation

Serum and plasma samples have to be diluted to 1:100 or 1:500 before performing the assay

### Test Procedure

1. Reconstitute Antibody, wash buffer and dilute plasma
2. Wash strips 5 x 250µl ELISA wash buffer
3. Add 100µl of standards, sample or control into wells
4. Incubate for 1 hour, covered and shaking on a horizontal mixer
5. Aspirate the wells and wash with wash buffer 5 x 250µl
6. Add 100µl of prediluted Peroxidase labeled CRP Antibody
7. Incubate for 1 hour, covered and shaking on a horizontal mixer
8. Decant the content of the plate and wash with wash buffer 5 x 250µl
9. Add 100µl of TMB Substrate into each well
10. Incubate for 5-10 mins at room temp in the dark
11. Add 50µl of stop solution then mix
12. Measure absorbance at 450nm



## 2.11 Thiobarbituric Acid Reactive Substances

### Reagents Required

1. 10% TCA                      0.815g TCA in 50ml of dw
2. 8.1% SDS                    0.810g SDS in 10ml of dw (wear mask)
3. 20% Acetic Acid pH=3.5
4. 0.8% TBA                    0.400g TBA in 50ml of dw
5. 1M TMP                      165 $\mu$ l TMP in 1ml ethanol
6. 1N-butanol

### Standard

1M TMP doubling dilutions from 10 $\mu$ M

### Plasma

1. add 0.75ml plasma and 0.75ml 10% TCA
2. boil for 30 minutes at 95 $^{\circ}$ C
3. cool with tap water
4. spin @ 3000 rpm for 10 mins

### Procedure

1. add 300 $\mu$ l of supernatant
  - i. 150 $\mu$ l of SDS
  - ii. 150 $\mu$ l of Acetic Acid
  - iii. 300 $\mu$ l of TBA
  - iv. 600 $\mu$ l of dw
  - v. (total volume = 1.5ml)
2. boil @ 95 $^{\circ}$ C for 60 mins
3. add 1.5ml 1-N-butanol
4. vortex then centrifuge @ 2500 rpm for 10mins
5. remove organic layer
6. read with fluri-meter excitation @515nm, emission @553nm

## 3.1 Reproducibility Study Inclusion / Exclusion Criteria

### 3.1.1 General

- Subjects must be willing and able to attend the clinic and undergo procedures as *per* the study protocol.
- Subjects must voluntarily give written informed consent to participate in the study. The investigator will be responsible for obtaining written informed consent before initiating any screening activities/procedures.
- At screening, subjects must be able to complete exhaled breath testing such that  $\geq 1$  ml of exhaled breath condensate (EBC) is collected after 10-15 minutes tidal breathing. Subjects must also be able to complete sputum induction with production a satisfactory sputum induction sample (defined as adequate sputum for differential cell count and collection of  $\geq 400\mu\text{l}$  of supernatant).

### 3.1.2 COPD patients

- Male or female out-patients 40-80 years of age with a documented diagnosis of COPD as defined by the GOLD guidelines [1].
- No evidence of significant reversibility to inhaled salbutamol; significant reversibility is defined as  $>12\%$  improvement in  $\text{FEV}_1$  following salbutamol.
- Clinically stable COPD i.e. no history of COPD exacerbation or respiratory tract infection within 1 month of screening **and** no history of hospitalisation for COPD within 2 months of screening **and** no greater than 2 COPD exacerbations within 6 months of screening (for the purposes of this study, an exacerbation is defined as a change in baseline dyspnoea, cough and/ or sputum production beyond day-to-day variability sufficient to merit a change in management).
- COPD patients must be current cigarette smokers or ex-smokers (defined as subjects who have given up smoking for  $\geq 6$  months) with a minimum of 10 pack years of tobacco exposure.
- Post-bronchodilator spirometry at screening showing:  $\text{FEV}_1/\text{FVC} < 0.7$  **and**  $\text{FEV}_1$  is  $< 80\%$  but  $> 30\%$  predicted for sex, age and height

### 3.1.3 Healthy volunteers

- Male or female volunteers aged  $\geq 40$  years
- For the “healthy smoker” subgroup, subjects will be current smokers with a smoking history of  $\geq 10$  pack years; these subjects will be age-matched as closely as possible ( $\pm 10$  years) to subgroup of “smokers with COPD”.
- For the “healthy ex-smokers” subgroup, subjects will be ex-smokers with a smoking history of  $\geq 10$  pack years, having not smoked for at least 6 months prior to screening; these subjects will be age-matched as closely as possible ( $\pm 10$  years) to “ex-smokers with COPD”.
- For the “non/never-smokers” subgroup, subjects will either have never smoked or have a smoking history of  $<1$  pack year history having not smoked for at least 5 years; attempts will be made to recruit subjects within a similar age range as the COPD population (smokers and ex-smokers) studied.
- All healthy subjects (smokers and non-smokers) must have spirometry (without bronchodilator administration) showing both FEV<sub>1</sub> and FVC of  $> 80\%$  predicted and FEV<sub>1</sub>/FVC ratio of  $>0.7$ .
- Volunteers must be free from clinically significant disease. A clinically significant disease is defined as any condition which the investigator considers could compromise either the volunteer’s safety or the validity of the study results.

### 3.1.4 EXCLUSION CRITERIA

- Subjects who are currently suffering from, or who have a past history (childhood or adult) of, a clinically significant atopic/allergic disorder (including seasonal allergic rhinitis, hayfever, asthma, eczema/dermatitis).
- Subjects who have a current or previous history of:
  - any upper respiratory tract inflammatory disease e.g. rhinitis, sinusitis ( $>1$  episode).
  - any lower respiratory tract disease (other than COPD) e.g. asthma, bronchiectasis, pneumoconiosis, sarcoidosis, bronchiolitis, lung cancer, tuberculosis.
  - an upper or lower respiratory tract infection within 1 month of screening
  - unexplained chronic cough/wheeze/dyspnoea.
  - any upper gastrointestinal tract inflammatory disorder (e.g. pharyngitis, oesophagitis, gastritis).
- Subjects with an unexplained chest radiograph opacity.

- Subjects who have received any experimental (i.e. non-marketed) drug within four months of screening, or who have undergone sputum induction or bronchoscopy within 1 month of screening.
- Screening sputum differential eosinophil count  $\geq 3\%$ .
- Subjects who have donated blood within 1 month of screening date, or subjects who have a date for blood donation falling within the prospective study period.
- Existence of any surgical or medical condition, as evident from medical history, direct questioning or physical examination which in the judgment of the Investigator might interfere with the conduct or results of the study.
- In the opinion of the Investigator, a subject who is not likely to complete the study for whatever reason.

### **3.1.5 Study Restrictions**

#### **3.1.5.1 Concomitant medications**

- The following medications will not be permitted during the study (subjects prescribed these medications must have stopped them for the indicated period prior to screening to be eligible):
  - Oral corticosteroids 8 weeks
  - Leukotriene modulators (zafirlukast, montelukast) 8 weeks
  - Nedocromil sodium or cromolyn sodium 8 weeks
- Subjects will be advised to take their usual bronchodilator medication, at the usual time of day, prior to attending the Unit (screening and study visits).
- Inhaled corticosteroids will be allowed throughout the study provided the dose remains constant.
- Subjects who require antibiotics (for any indication) should undergo screening no less than 4 weeks after their final antibiotic dose and/or termination of symptomatology necessitating the course. Subjects deemed to require antibiotics during the study will be withdrawn and may not be re-entered.
- Subjects who are due to receive influenza or *S. pneumoniae* vaccination are not eligible for the study if vaccination is due within 2 weeks of prospective screening date, or between screening and visit 2.

#### **3.1.5.2 Caffeine**

Subjects should refrain from ingesting caffeinated products (including tea, coffee) for at least 8 hours prior to attending for screening or study visits.

#### **3.1.5.4 Smoking**

COPD patients and “healthy” subjects who smoke will be required to abstain from smoking for a minimum of 8 hours prior to attending the Investigational Unit for screening or study visit procedures.

#### **3.1.5.5 Activity/exercise**

Subjects should refrain from strenuous exercise for at least 24 hours prior to screening or study visits.

#### **3.1.5.6 Stability criteria**

Any subject who experiences a respiratory tract infection and/or any clinically significant deterioration of their COPD symptoms between screening and study visit 1 will not be entered at that point. At the discretion of the Investigator, these subjects may be re-screened, but re-screening may take place no less than 4 weeks following full resolution of the infection/exacerbation and return to their usual therapeutic regimen.

If subjects experience a respiratory tract infection or any clinically significant deterioration of their COPD symptoms in the period between study visit 1 and study visit 2, then they will be withdrawn from the study and may not be re-entered.

Even in the absence of overt respiratory tract infection or clinically significant deterioration in symptoms, if the FEV<sub>1</sub> measured for COPD patients or healthy subjects on the study day is outside the range of 90-115% of the pre-bronchodilator value at screening visit, they will not complete study procedures (including exhaled breath collection, sputum induction and venesection) on that day. However, assuming that no other evidence of a potential exacerbation is observed and no alteration in the subject's medication is deemed necessary by the attending physician, the visit may be rescheduled to occur 3-7 days thereafter. Failure to obtain an FEV<sub>1</sub> value within the permissible range at this rescheduled visit will result in withdrawal of the patient from the study.

## Appendix 4.1 Cross sectional Study Inclusion / Exclusion Criteria

### 4.1.1 General

- Subjects must be willing and able to attend the clinic and undergo procedures as *per* the study protocol.
- Subjects must voluntarily give written informed consent to participate in the study. The investigator will be responsible for obtaining written informed consent before initiating any screening activities/procedures.
- At screening, subjects must be able to complete exhaled breath testing such that  $\geq 1$  ml of exhaled breath condensate (EBC) is collected after 10-15 minutes tidal breathing.

### 4.1.2 COPD patients

- Male or female out-patients 40-80 years of age with a documented diagnosis of COPD as defined by the GOLD guidelines [1].
- No evidence of significant reversibility to inhaled salbutamol; significant reversibility is defined as  $>400$ ml improvement in  $FEV_1$  following salbutamol.
- Clinically stable COPD i.e. no history of COPD exacerbation or respiratory tract infection within 1 month of screening **and** no history of hospitalisation for COPD within 2 months of screening **and** no greater than 2 COPD exacerbations within 6 months of screening (for the purposes of this study, an exacerbation is defined as a change in baseline dyspnoea, cough and/ or sputum production beyond day-to-day variability sufficient to merit a change in management).
- COPD patients must be current cigarette smokers or ex-smokers (defined as subjects who have given up smoking for  $\geq 6$  months) with a minimum of 10 pack years of tobacco exposure.
- Post-bronchodilator spirometry at screening showing:  $FEV_1/FVC < 0.7$  **and**  $FEV_1$  is  $< 80\%$  predicted for sex, age and height

### 4.1.3 Healthy volunteers

- Male or female volunteers aged  $\geq 40$  years
- For the “healthy smoker” subgroup, subjects will be current smokers with a smoking history of  $\geq 10$  pack years; these subjects will be age-matched as closely as possible ( $\pm 10$  years) to subgroup of “smokers with COPD”.
- For the “healthy ex-smokers” subgroup, subjects will be ex-smokers with a smoking history of  $\geq 10$  pack years, having not smoked for at least 6 months prior to screening; these subjects will be age-matched as closely as possible ( $\pm 10$  years) to “ex-smokers with COPD”.

- For the “non/never-smokers” subgroup, subjects will either have never smoked or have a smoking history of <1 pack year history having not smoked for at least 5 years; attempts will be made to recruit subjects within a similar age range as the COPD population (smokers and ex-smokers) studied.
- All healthy subjects (smokers and non-smokers) must have spirometry (without bronchodilator administration) showing both FEV<sub>1</sub> and FVC of > 80% predicted and FEV<sub>1</sub>/FVC ratio of >0.7.
- Volunteers must be free from clinically significant disease. A clinically significant disease is defined as any condition which the investigator considers could compromise either the volunteer’s safety or the validity of the study results.

#### 4.1.4 EXCLUSION CRITERIA

- Subjects who are currently suffering from, or who have a past history (childhood or adult) of, a clinically significant atopic/allergic disorder (including seasonal allergic rhinitis, hayfever, asthma, eczema/dermatitis).
- Subjects who have a current or previous history of:
  - any upper respiratory tract inflammatory disease e.g. rhinitis, sinusitis (>1 episode).
  - any lower respiratory tract disease (other than COPD) e.g. asthma, bronchiectasis, pneumoconiosis, sarcoidosis, bronchiolitis, lung cancer, tuberculosis.
  - an upper or lower respiratory tract infection within 1 month of screening
  - unexplained chronic cough/wheeze/dyspnoea.
  - any upper gastrointestinal tract inflammatory disorder (e.g. pharyngitis, oesophagitis, gastritis).
- Subjects with an unexplained chest radiograph opacity.
- Subjects who have received any experimental (i.e. non-marketed) drug within four months of screening, or who have undergone sputum induction or bronchoscopy within 1 month of screening.



## **4.1.5 Study Restrictions**

### **4.1.5.1 Caffeine**

Subjects should refrain from ingesting caffeinated products (including tea, coffee) for at least 8 hours prior to attending for screening or study visits.

### **4.1.5.2 Smoking**

COPD patients and “healthy” subjects who smoke will be required to abstain from smoking for a minimum of 8 hours prior to attending the Investigational Unit for screening or study visit procedures.

## **4.2 MRC Dyspnoea Score**

- 0 Not troubled with breathlessness except with strenuous exercise
- 1 Troubled by shortness of breath when hurrying or walking up a slight hill
- 2 Walks slower than people of the same age due to breathlessness or has to stop for breath when walking at own pace on the level
- 3 Stops for breath after walking about 100m or after a few minutes on the level
- 4 Too breathless to leave the house or breathless when dressing or undressing



### 4.3 Past Medical History in Cross Sectional Study Group

Study Number	Study Group	Smoking status	Other Medical History
L220	1	2	Pancreatitis colonic polyp
L030	1	2	PVD
L067	1	2	Peptic ulcer disease Hypocalcaemia
L071	1	2	Schizophrenia
L073	1	2	psoriasis anxiety , vocal cord polyps Sigmoid colectomy Non specific shoulder pain
L076	1	2	OA Hiatus hernia Leg cramps
L077	1	2	cervical spondylosis
L082	1	2	Appendicectomy
L090	1	2	Knee op Slipped disc
L091	1	2	Chronic Schizophrenic Hearing impairment
L092	1	2	PVD intermittent claudication
L099	1	2	
L100	1	2	
L101	1	2	Renal Colic Restless leg syndrome occasional headache
L102	1	2	
L104	1	2	PVD AFHypotension gout
L105	1	2	cirrhosis TURP Lumber discopathy
L106	1	2	Congenital nystagmus Childhood pneumonia
L109	1	2	Sub Arachnoid Haemorrhage Angina Chronic back pain OA Depression PVD
L114	1	1	Depression
L119	1	1	Diptheria Atopy to metals heart murmur
L120	1	2	truncal Vagotomy & Pyloroplasty for Duodenal Ulcer

L126	1	2	Depression
L129	1	2	CABG
L130	1	1	
L132	1	2	BPH angioplasty and stenting
L133	1	1	BPH Cateract op
L134	1	2	PVD
L136	1	2	Sterilization
L137	1	2	Ventilated March 2005
L140	1	2	Dupuytrens contracture tonsilectomy
L148	1	2	Anxiety peptic ulcer disease bowel resection
L149	1	2	pneumonia & pleurisy age 12 prostatectomy
L151	1	2	Trochanteric Bursitis Left fractured neck of femur
L152	1	2	
L154	1	2	Cerebral Aneurysm clipped Cervical spondylosis
L155	2	1	AF eczema
L157	2	1	Gout pancreatitis diverticulitis
L158	2	1	Diverticulitis EMD arrest
L160	2	1	MI Depression
L162	2	1	
L163	2	1	BPH Cataracts Angina.
L164	2	1	Diverticular Disease
L166	2	1	CABG Sciatica unstable bladder inguinal hernia
L167	2	1	
L168	2	1	PVD Dyspepsia OA
L170	2	1	TIA Dyspepsia
L173	2	1	Bilateral Hip Replacement
L174	2	1	Pneumothorax

L175	2	1	DU Cholecystectomy
L176	2	1	Previous DVT
L178	2	1	Hypertension
L179	2	1	MI 25yrs ago Gout.
L180	2	1	Hernia op
L181	2	1	Colectomy for Colonic ca Post op pneumothorax
L186	2	1	
L187	2	2	Depression
L188	2	1	TIA esophagitis
L189	2	1	
L190	2	1	Atrial flutter Prostatectomy for Ca 1988
L191	2	1	Glaucoma
L193	2	1	Enlarged prostate
L194	2	1	Trigeminal Neuralgia
L195	2	1	Osteoarthritis
L196	2	1	BPH
L197	2	1	OSA Vertebro-basilar insufficiencyOA
L198	2	1	Depression dyspepsia reflux
L199	2	1	Non hodgkins lymphoma 1995
L201	2	1	CABG
L202	2	1	Pleurisy age 18 mastoid op hypercholesterolaemia
L204	2	1	Hiatus Hernia duodenal ulcer
L205	2	1	BPH
L206	2	1	
L209	2	1	
L210	2	1	hypothyroidism osteoarthritis
L212	2	1	benign prostatic hypertrophy lumbosacral spondylosis

L213	2	1	Hypercholesterolaemia TIA 2000
L214	3	2	
L215	3	2	
L217	3	2	
L218	3	2	
L219	3	2	
L221	3	2	
L222	3	2	
L223	3	2	
L224	3	2	
L225	3	2	
L226	3	2	
L227	3	2	
L228	4	1	Contact dermatitis Pneumonia 1965 OA
L229	4	1	varicose veins Rosacea
L230	4	1	acne rosacea
L231	4	1	AF
L232	4	1	allergy to penicillin sinus problems
L233	4	1	Anaphylaxis to penicillin
L234	4	1	Back Injury - slipped L4 L5
L235	4	1	Bhronchitis/asthma as child Pylori oct 2004
L236	4	1	Dupuytren peumonia aged 12 anaemic
L237	4	1	Bowen's disease Allergy to iodine hypothyroidism
L238	4	1	bronchitis 10yrs ago
L241	4	1	Bronchitis age 19 no recurrence Varicose Veins
L243	4	1	bronchitis aged 22
L244	4	1	CABG 1992

L245	4	1	CABG MI BPH OA Cataract op PAF
L246	4	1	carpal tunnel syndrome
L247	4	1	Cholecystectomy Irritable Bowel Syndrome
L248	4	1	Depression OA osteoporosis Pneumonia
L249	4	1	Depression late 1990's
L250	4	1	dyspepsia lower back pain hayfever
L251	4	1	Frozen Shoulder
L252	4	1	Gout
L253	5	0	H. pylori Gastritis
L255	5	0	hiatus hernia GORD psoriasis – uv treated
L256	5	0	Hypercholesterolaemia - diet controlled
L258	5	0	Hypertension not medicated
L259	5	0	Hypothyroid
L260	5	0	Hypothyroid
L262	5	0	Kidney probs unknown cause
L263	5	0	L hip bursitis 2002
L264	5	0	Left nephrectomy acute arthritis
L265	5	0	MI 1981 CABG 1991
L267	5	0	MI 2002 angioplasty Migraine Diverticular disease
L268	5	0	MI eye surgery Colostomy for colonic abscess - reversed TURP for benign prostatic renal calculi.
L269	5	0	Migraine Psoriasis gluten intolerance
L270	5	0	Migraines
L272	5	0	Mild OA
L273	5	0	Neuromas sciatica hysterectomy GORD
L275	5	0	Nil
L276	5	0	Nil
L280	5	0	pneumonia 1972 nasal polyps (op 2004)

L281	5	0	Pneumonia age 14
L282	5	0	possible Erythromycin allergy
L283	5	0	Recurrent back problems
L285	5	0	sinusitis 6weeks ago
L287	5	0	Varicose Veins D+C benign breast cysts

**Study Groups**      1 = COPD smoker, 2= COPD ex-smoker, 3 = healthy smoker,  
4 = healthy ex-smoker, 5 = healthy never smoked

**Smoking Status**      0 = never, 1= ex-smoker, 2 = current smoker

## 5.1 Nacystelyn Study Schedule

Period	Screenin	Double Blind Treatment				Follow-up
Visit	1	2	3	4	5/ET	6
Week	-	0	2	4	12	14
Day	-31 to -14	0	14	28	84	98
Visit Window (Days)	-	-	(±3)	(±3)	(±3)	(±3)
Informed Consent	X					
Demography	X					
Medical History	X	X				
Inclusion/Exclusion Criteria	X	X				
Randomisation		X				
Clinical Examination	X	X	X	X	X	X
SGRQ		X		X	X	
Symptom Score Patient Diary Completion	X		X	X	X	
Reversibility Testing	X					
Pulmonary Function Tests †	X	X	X	X	X	X
Electrocardiogram (ECG)	X					
Concomitant Treatments	X	X	X	X	X	X
Training & Assessment on use of Inhaler	X					

Device						
Dispense (x) / Retrieve (o) Placebo Run-In Medication	x	o				
Dispense (x) / Retrieve (o) Study Medication		x		x o	o	
Dispense (x) / Retrieve (o) fluticasone	x	X o		x o	o	
Haematology & Serum Chemistry	X			X	X	
Serology (Screening for Hep. B and Hep. C)	X					
Urinalysis (* Including drug screening)	X*			X	X	
Serum Pregnancy Test	X					
Lipid Peroxidation in Plasma	X			X	X	
Breath Condensate		X		X	X	
Sputum Samples (Induced)		X		X	X	
Sputum Samples (Spontaneous)			X			X
Adverse Events / Serious Adverse Events		X	X	X	X	X

†: Pulmonary function tests (forced expiratory volume [FEV<sub>1</sub>], forced vital capacity [FVC], and peak expiratory flow rate [PEFR]) were performed both pre and post bronchodilator inhalation according to a standard procedure. Patients were instructed not to take short acting  $\beta$ 2-agonists at least 8 hours, long acting  $\beta$ 2-agonists at least 24 hours, and inhaled anticholinergics at least 8 hours prior to each study visit.

## 5.2 Nacystelyn Study Inclusion / Exclusion Criteria

### *Subject Inclusion Criteria*

The patients who may be included in the trial are those:



- Who are cooperative male or female outpatients aged  $\geq 40$  years
- Who are smokers or ex-smokers of 10 pack years or more.
- With COPD according to the ERS guidelines<sup>43</sup>, with an FEV<sub>1</sub> between 30% and 60% predicted and who demonstrate  $<15\%$  reversibility from baseline and  $\leq 200$  ml improvement in FEV<sub>1</sub> after inhalation of 2.5 mg nebulised salbutamol.
- Who have chronic symptoms, i.e. chronic breathlessness with or without cough and sputum production.
- Who have had at least 1 respiratory exacerbation (defined as seeking medical advice and changing treatment to oral antibiotics and/or oral steroids) during the winter season prior to the study.
- Who can properly use the MIAT monodose DPI.
- Who have signed a written informed consent.

### ***Subject Exclusion Criteria***

#### ***At Screening***

- Patients who have any history of asthma.
- Patients who are clinically unstable, i.e. who have had a respiratory exacerbation  $\leq 4$  weeks before the start of the trial.
- Patients with unstable concurrent non-respiratory diseases.
- Patients with active lung disease other than COPD.
- Patients with, or at risk of contracting, tuberculosis, HIV, Hepatitis B or Hepatitis C.
- Known hypersensitivity to nacystelyn or fluticasone.
- Patients who are intolerant of lactose.
- History of non-compliance to medical regimens and patients who are considered potentially unreliable.
- Treatment with any investigational drug within the past month or 10 half lives, whichever is longer.
- History of malignancy of any organ system, treated or untreated, within the past five years whether or not evidence of local recurrence or metastases exists are excluded, with the exception of localised basal cell carcinoma of the skin.
- Patients involved in disease-related litigation.
- Women who are pregnant or lactating

### **Subject Withdrawal Criteria**

The patient will be advised in the Informed Consent Forms that they have the right to withdraw from the study at any time without prejudice, and may be withdrawn at the Investigator's/Chiltern's/Laboratoires SMB's discretion at any time. Prematurely discontinued patients will not be replaced.

In the event that the patient drops out of the study or is withdrawn from the study, the patient should undergo an early termination visit. All Visit 5/ET procedures should be carried out as required and the Investigator should record the details of the withdrawal on the study completion page of the CRF. If possible the patient should also return for the follow up visit 14 days after their ET visit to have a clinical examination and pulmonary function tests carried out. Where this is not possible a follow up telephone call should be made to the patient to determine if any SAEs have occurred since the termination of study drug. (N.B. All SAEs occurring up to 30 days after a patient stops taking the study medication should be recorded and reported as detailed in section 9.3 of this protocol).

Reasonable effort should be made to contact any patient lost to follow up during the course of the study in order to complete assessments and retrieve any outstanding data and study medication/supplies.

The following are reasons for patient dropout/withdrawal:

- Adverse event
- Protocol deviation (e.g. dosing regime, failure to comply with clinic visit schedule)
- Administration of an excluded medication
- Co-existing disease
- Worsening of condition
- Pregnancy
- Clinical significant abnormal laboratory value(s)
- Other

The patient requested withdrawal due to:

- An adverse event for which the Investigator did not consider removal from the study necessary.
- Perceived insufficient therapeutic effect.
- Co-existing disease
- Withdrawal of consent
- Other

Other

- Patient died
- Lost to follow-up
- Administrative problems

## 5.4 Nacystelyn Study Patient Diary Symptom Score

	e.g.	Day 1	Day 2	Day 3	Day 4
Date: dd / mm	15/07	/	/	/	/
1. How many times did you awake during last night because of your chest problems? <i>(enter number of times)</i>	1				
2. How breathless have you been in the last 24 hours? Score (0-4) <i>(circle appropriate score)</i>	0	0	0	0	0
	1	1	1	1	1
	2	2	2	2	2
	3	3	3	3	3
	4	4	4	4	4
3. How much have you coughed in the last 24 hours? Score (0-3) <i>(circle appropriate score)</i>	0	0	0	0	0
	1	1	1	1	1
	2	2	2	2	2
	3	3	3	3	3
4. How much sputum have you produced in the last 24 hours? Score (0-3) <i>(circle appropriate score)</i>	0	0	0	0	0
	1	1	1	1	1
	2	2	2	2	2
	3	3	3	3	3
5. What colour has your sputum been in the last 24 hours? Score (0-4) <i>(circle appropriate score)</i>	0	0	0	0	0
	1	1	1	1	1
	2	2	2	2	2
	3	3	3	3	3
	4	4	4	4	4
6. Have you missed any time from work or usual activities in the last 24 hours because of your chest problems? <i>(circle appropriate answer)</i>	None	None	None	None	None
	Half day	Half day	Half day	Half day	Half day
	Full day	Full day	Full day	Full day	Full day
7. Have you needed any assistance from a caregiver (paid or unpaid) in the last 24 hours because of your chest problems? <i>(circle appropriate answer)</i>	None	None	None	None	None
	Half day	Half day	Half day	Half day	Half day
	Full day	Full day	Full day	Full day	Full day

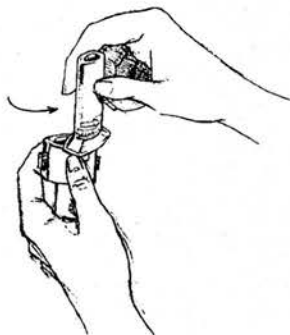
# Appendix 5.5

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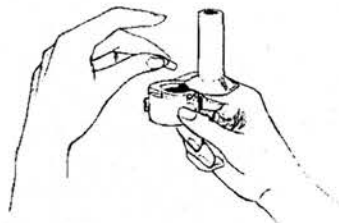
## INSTRUCTION FOR USE



1. Remove the cap.



2. Steadily hold the base and open the inhaler turning the nozzle anti-clockwise (see the arrow on the nozzle).



3. Introduce the capsule into the loading chamber.



5. Firmly press the two red buttons, keeping the inhaler in the vertical position.



6. Breathe out deeply.



7. Introduce the whole nozzle into the mouth and breath in, closing the lips (avoid air passing around the nozzle) with the head bent backwards. Hold your breath for 3-5 seconds. Repeat until the capsule is completely empty.